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MAARIT NIEMI

FECAL INDICATOR BACTERIA AT FRESHWATER RAINBOW TROUT *(SALMO GAIRDNERI)* FARMS

Tiivistelmä

Fekaali indikaattoribakteerit sisävesien kirjolohen kasvatuslaitoksilla

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The occurrence of fecal indicator bacteria, such as fecal streptococci, total coliforms and thermotolerant coliforms, was measured at rainbow trout farms during the growing season. Multiplication of presumptive fecal streptococci and total coliforms at the farms was considerable. However, confirmation with bile esculin azide agar at 44 °C and tolerance tests revealed that the increase of presumptive fecal streptococci was due to non-fecal strains. Only a minority of total coliform bacteria were *Escherichia coli*, whereas the vast majority of thermotolerant coliforms were *E. coli*. These bacteria only occasionally increased at fish farms. These results were interpreted as an indication of fecal contamination of runoff waters at the farm. The contamination was probably due to bird droppings.

Coliform bacteria were identified using the API 20 E system. *Citrobacter, Aeromonas hydrophila* and *E. coli* were the major taxa. However, many of the isolates remained unidentified and the reliability of the identification to *Citrobacter freundii* was questionable. Numerical taxonomy was therefore applied to this material.

Index words: fish farms, *Salmo gairdneri*, fecal indicator bacteria, fecal streptococci, total coliforms, thermotolerant coliforms, *Escherichia coli, Aeromonas hydrophila, Citrobacter*, numerical taxonomy, API 20 E.

1. INTRODUCTION

1.1 Fish farming and water quality

Fish farming is increasing rapidly throughout the world and Finland is no exception. In 1982, 296 freshwater farms and hatcheries, 203 natural rearing pond breeders and 98 brackish water cage farms produced fish intended for human comsumption and for the improvement of fisheries by transplantation (Eskelinen and Sumari 1983). The brackish water cage farms produced 3200 tons and the freshwater farms 3100 tons of fish for human comsumption. The fish produced was almost exclusively rainbow trout (*Salmo gairdneri*).

Aquaculture is an economic means of producing animal protein with low energy costs. The Food and Agriculture Organization of the United Nations promotes the development of aquaculture in forms less dependent on the support of industrial technology (Ackefors and Rosén 1979). Even in the western world, fish farming is still predominantly carried out on a relatively primitive basis, intensive but without effluent treatment and water circulation. Inevitably such activities will run into environmental difficulties once they are conducted in a sufficiently large scale.

In Finland the tendency in rainbow trout farming is towards large farms situated in relatively

unpolluted watercourses or sea areas. This has provoked considerable public concern over water pollution problems. The general public has complained of the deterioration of water quality due to fish farming and the Water Authorities have collected information on the effects of fish farms on water quality. A committee report on the environmental effects of fish farming in Finland has been published (Kalanviljelyn ympäristöhaittatoimikunta 1982). The main cause of concern usually is eutrophication but other problems e.g. the increase of indicator bacteria of water hygiene, have been observed (Niemi and Taipalinen 1982, Sumari 1982).

In the United Kingdom the effects of fish farm effluents on freshwater quality were reported by Solbe (1982). Causes for concern were the abstraction of a large part of the flow of the river, the large size of some farms, the location of series of several farms along one stretch of river, and the use of antibiotics and antibacterial agents without sufficient knowledge of their effects on wild fish populations, other farmed fish, or humans. Further causes for concern were reduction in dissolved oxygen concentration and increases in biochemical oxygen demand (BOD), suspended solids, phosphorus, ammonia, nitrite and nitrate. The increases in phosphorus and nitrogen were evaluated as problematical for oligotrophic waters. Suspended solids were regarded as the most obvious cause of complaint, because the average fish farm discharges the same amount of solids as an efficient sewage works serving 21 000 people.

1.2 Indicators of water hygiene

The potential presence in water of pathogenic microorganisms excreted in the feces of humans and of homoiothermic animals is demonstrated indirectly by determining bacterial species of the normal flora of feces. The most important of such organisms are coliform bacteria and fecal streptococci.

The use of coliform bacteria as indicators of the hygienic state of water originates from the last century. The routine methods of enumeration allow the growth of Gram-negative, non-sporing rods that are oxidase negative, aerobic or facultatively anaerobic, cabable of growth in the presence of bile salts or other surface active agents and able to ferment lactose with the production of acid, gas and aldehyde within 48 hours between 35 and 37 °C. This definition of coliform bacteria, which includes lactose fermenting genera and species of the family *Enterobacteriaceae*, was prepared by the water committee of the International Organization of Standardization (ISO/TC 147/SC 4/WG 2 N 29 E 1983). In routine water analyses not all of these characteristics are tested. The primary cultivation on plates is not selective enough to prevent the growth of oxidase positive *Aeromonas*-species, which are common in the aquatic environment (Mack 1977).

The selectivity of the method has been improved by increasing the incubation temperature (Eijkman 1904, Geldreich et al. 1965). The enumeration of thermotolerant coliform bacteria selects mainly the genera *Escherichia* and *Klebsiella*, with occasionally other genera (Dufour 1977, Niemelä et al. 1983). Dufour recommended methods that detect *E. coli* after primary cultivation at the elevated temperature followed by biochemical tests to distinguish *E. coli*. These procedures are used in Belgium, Denmark, France and the United Kingdom. The routine methods for thermotolerant coliform bacteria used in North America, Norway, Sweden and Finland allow the growth of *E. coli* and of the other coliform bacteria, mainly *Klebsiella*. The genus *Klebsiella* is taken as an indicator of wastes high in organic matter and of human activities, but not as an indicator of fecal contamination (Vlassoff 1977). Additional identification tests to distinguish *E. coli*, which is considered as the true fecal indicator (Dufour 1977, Cabelli 1978), from other thermotolerant coliform bacteria are needed in order to verify the source of pollution.

Fecal streptococci include *Streptococcus faecalis*, *S. faecium*, group Q streptococci, *S. bovis*, *S. equinus*, *S. mitis* and *S. salivarius* (Levin et al. 1975, Clausen et al. 1977, Beaudoin and Litsky 1981). Of these, *S. faecalis* and *S. faecium* are usually regarded as enterococci, although *S. avium* (group Q) also conforms to the Sherman criteria of enterococci. The enterococci, together with *S. bovis* and *S. equinus*, belong to group D streptococci. Human feces are characterized by a great predominance of enterococci. *S. bovis* and *S. equinus* are rarely recovered from human feces but are typical to the feces of livestock. *S. mitis* and *S. salivarius* are buccal streptococci which occur in low numbers in human feces but not in animal feces.

The fecal streptococci occur in feces in lower concentrations than coliform bacteria except in swine feces in which they are abundant (Geldreich 1966). As well as in intestines, fecal streptococci can also multiply in plants (Clausen et al. 1977, Beaudoin and Litsky 1981). Their survival is often better than that of coliforms. Beaudoin and Litsky (1981) pointed out that fecal coliforms are not a satisfactory indicator under all conditions and that

it is often advisable to consider both fecal coliform and fecal streptococcus densities when determining the hygienic quality of water.

1.3 Bacteria in fish and at fish farms

In natural undisturbed conditions the microflora of fish skin reflects both quantitatively and qualitatively the aquatic environment (Suominen 1980). On the basis of a bibliographic analysis Lesel (1981) concluded that the composition of the microflora of the digestive tract of fish depends on that of the external environment and of the ingested food. A supply of food gives rise to intense multiplication of the bacteria of the alimentary tract, which differ in their biochemical characteristics from the aquatic strains.

A rather wide variety of aerobic and anaerobic bacteria have been isolated from the intestinal contents of free-living and cultured freshwater fish (Trust and Sparrow 1974, Souter et al. 1976, Yoshimizu et al. 1976, Allen et al. 1983, Sugita et al. 1983). The dominant flora consists of *Enterobacteriaceae, Aeromonas-Vibrio, Pseudomonas, Bacillus,* coryneform bacteria, *Flavobacterium* and *Achromobacter. Micrococcus* was dominant in pond fish in the Ukraine (Antipchuk 1975). Rodshaped and coccal forms of lactic acid bacteria dominated, up to 10^8 CFU (colony forming unit) per gram, in the intestinal contents of carp (*Cyprinus carpio*) in the same area (Kvasnikov et al. 1977). Sugita et al. (1983) found 10^4 to 10^8 CFU g^{-1} of *Enterobacteriaceae* and the *Aeromonas-Vibrio* group in fish from a Japanese river.

Fecal indicator bacteria such as fecal streptococci, total coliforms and thermotolerant coliforms have been isolated from free living and cultured fish (Geldreich and Clarke 1966, Trust and Sparrow 1974, Yoshimizu et al. 1976, Kvasnikov et al. 1977). Ruane et al. (1977) observed 10^5 CFU of thermotolerant coliform bacteria per millilitre in the effluent from a catfish farm.

E. coli (Watanabe et al. 1971, Trust and Sparrow 1974, Aoki 1975, Souter et al. 1976), *Salmonella* (Souter et al. 1976) and *Shigella* (Trust and Sparrow 1974) have been isolated from fish intestines.

1.4 Aims of the study

The main objectives of this study were to evaluate the amounts of total and thermotolerant coliform bacteria and fecal streptococci introduced to water bodies by fish farms and to determine whether these belong to species of bacteria indicating fecal contamination.

The suitability of routine methods for measuring fecal contamination in samples of water affected by fish farm efluents is discussed, as well as the suitability of a commercially available identification test kit for coliform bacteria isolated from these samples.

2 MATERIALS AND METHODS

2.1 The fish farms

The observations on bacterial, chemical and physical water characteristics at 27 rainbow trout (*Salmo gairdneri*) farms between the beginning of May and the end of October were supplied by the Oulu, Kuopio, Tampere, Kajaani, Helsinki and Lappi Water District Offices. The material included more observations from larger farms than from smaller cultures (Table 1).

Table 1. The fish farms studied.

Fish farm	No. of sampling dates	No. of variables measured
Vääksy	44	8 – 9
Tyyrinvirta	30	5 — 9
Äyskoski	19	8 — 9
Längelmäki	15	8 — 9
Iijoen lohiyhtymä	7	8 — 9
Soivio	7	7 — 10
Myllyjoki	7	8
Kolvanki	6	8
Perjakka	5	8 — 9
Paahtojärvi	5	7 — 8
Joukamon Lohi	5	8
Suininki	5	8
Karkkilan Lohi	5	8
Laukasenniva	4	8
Varisjoen Lohiyhtymä	4	8
Tervasalmen Lohi	3	8
Salmijoen Lohi	3	8
Ylä-Kiannan Lohi	2	6 — 7
Hossanjoki	1	6
Paltalohi	1	6
Parkkilan Lohi	1	7
Yrittäperän Lohi	1	7
Kainuun Lohi	1	7
Eero Hakkarainen Firm	1	7
Lohela	1	7
Särkijärvi	1	6
Juutuanjoki	1	7

The two rainbow trout farms investigated most intensively were the farms of Savon Taimen Ltd. by the River Tyyrinvirta (Northern latitude 695280, longitude east from Greenwich 49150, Fig. 1) and of Nilakkalohi Ltd. by the rapids of Äyskoski (Northern latitude 698550, longitude east from Greenwich 48500, Fig. 2). Both of the farms are situated in the Rautalampi watercourse, which is not polluted by industrial effluents or appreciably by municipal effluents. The Tyyrinvirta farm is situated further down the watercourse than the Äyskoski farm.

The Tyyrinvirta farm produced about 400 tons and the Äyskoski farm about 300 tons of fish annually. Fish were grown in earthen raceways and pools and in addition at the Äyskoski farm 100 tons were grown in the river area in net enclosures. At the Tyyrinvirta farm only pelleted dry feed was used, whereas at the Äyskoski farm small amount of ground small fish was used in addition to the dry pellets. Feeding started at seven a.m. at both farms and continued throughout the working day. Once a week malachite green and formaline were applied to young fish at both farms.

The effluents from the Tyyrinvirta farm itself flowed untreated to the river. Domestic wastes and wastes from an associated furred animal farm and from the facilities for scaling and cutting fish were drained into the effluent from the rearing channel after purification. The effluent from the middle channel comprised on average 67 per cent and the effluent from the rearing channel 33 per cent of the effluent volume (Fig. 1).

About 60 per cent of the effluent at the

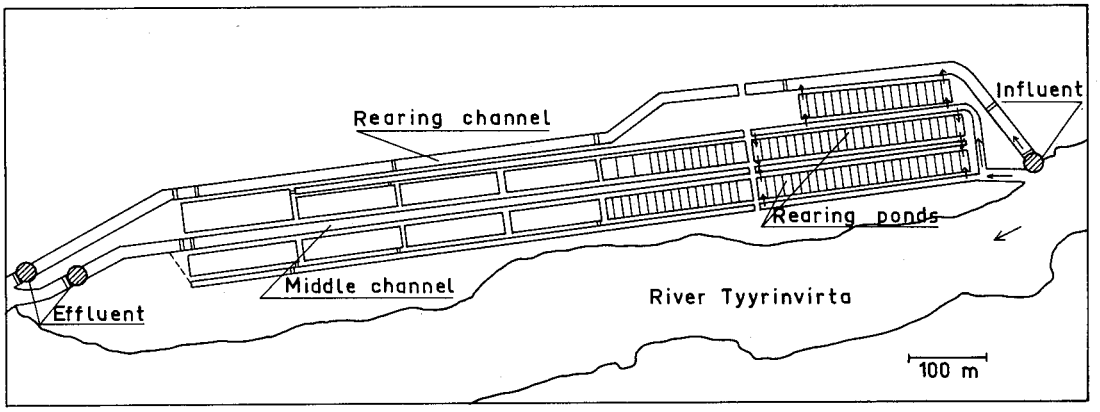

Fig. 1. The Tyyrinvirta farm.

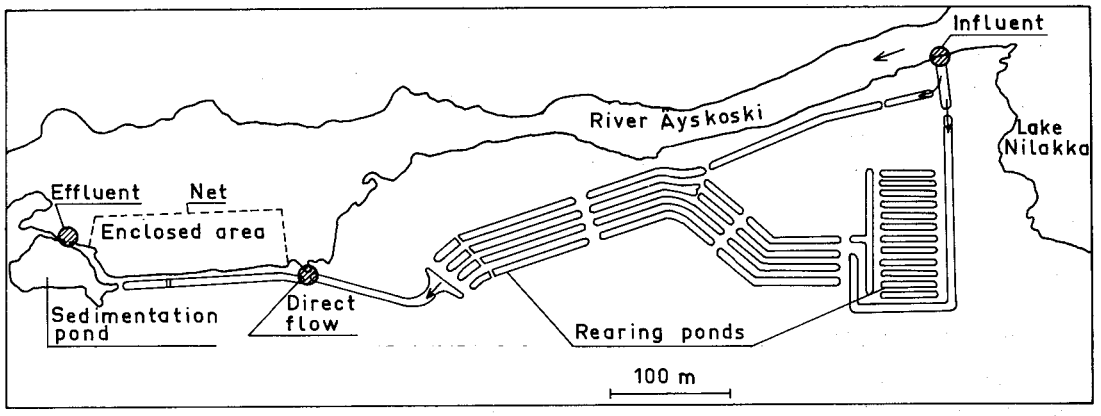

Fig. 2. The Äyskoski farm.

Äyskoski farm drained into the Äyskoski rapids after sedimentation, while 40 per cent was untreated or clarified with a swirl clarifier (Fig. 2).

2.2 Samples

For chemical and physicl analyses the samples were taken without replicates. For the enumerations of bacteria the majority of samples from the Tyyrinvirta and the Äyskoski farms were taken in duplicate.

Samples from the Tyyrinvirta and Äyskoski farms for bacterial analyses were taken from the influent and from two effluent fractions directly to sterile borosilicate bottles and from silt deposits on the raceway and pool bottoms (from two to three sites) with a clean spade. All samples were taken in duplicate. Duplicate samples were taken from dry pelleted feed and on one occasion from fresh feed. Two composite samples containing the intestinal contents of five fish each were collected. The fishes were killed and the abdominal cavity immediately opened with a clean knife, after which the intestinal contents were aseptically transferred into sterile borosilicate bottles.

Samples from the two farms were taken during the growing season usually on alternate weeks for bacterial, chemical and physical analyses. When possible, additional samples were also taken. In 1981 coliform bacteria were isolated for identification and in 1982 strains of fecal streptococci were isolated.

The samples for bacteriological enumerations were always analyzed within a few hours of sampling, except in the case of enumerations of bacteria from dry pelleted feed, which were carried out even weeks after sampling.

2.3 Hydrological, physical and chemical measurements

The Finnish Meteorological Institute provided information on rainfall at the Rastu and Vesanto stations. The fish farms supplied information on discharge. The influent and effluent water temperature was measured at sampling. Finnish standard methods were used to measure conductivity (SFS 3022, 1974), pH (SFS 3021, 1979), dissolved oxygen (SFS 3040, 1975), chemical oxygen demand (COD_{Mn}, SFS 3036, 1981) and ammonia-nitrogen (SFS 3032, 1976). Modifications of the Swedish standard methods were used to analyse total nitrogen (SIS 028131, 1976), total phosphorus (SIS 028127, 1974), and phosphate phosphorus (SIS 028126, 1974).

2.4 Bacteriological analyses

2.41 Enumeration techniques used

Water samples were shaken carefully before taking portions for the cultivation. Fecal and sediment samples were homogenized manually and pelleted feed with a Warring Blender before weighing. Samples were diluted into phosphate magnesium sulphate diluent (APHA 1980).

The filtration technique and Sartorius SM 13806 AC 37 membranes were used for water samples. The spread plate technique was used to enumerate coliform bacteria from sediments and fish feces. The pour plate technique was used for these samples in the enumeration of fecal streptococci. The MPN (most probable number) technique was applied in the enumeration of bacteria from pelleted fish feed. In the MPN procedure one bottle containing 100 millilitres of medium was inoculated with a ten gram feed sample, one series of ten tubes containing ten millilitre portions of medium with one gram samples, and one series of ten tubes containing ten millilitre portions of medium with one hundred milligram samples. The formula of Thomas (1942) was used for the calculation of the MPN.

2.42 Enumeration and identification of total coliform bacteria

For the colony counting techniques LES Endo agar (Difco) and incubation at 35±1°C for 22±2 hours were used (SFS 3016, 1984). The enumeration of total coliform bacteria with LES Endo agar was not satisfactory. Non-typical background colonies were so common that they outnumbered typical colonies. Therefore it was not always possible to count enough typical colonies to reach statistically satisfactory results.

In the MPN procedure lactose bromcresol purple broth and incubation at 35±1° for 44±4 hours were used for the primary fermentation. Brilliant green bile broth and the same incubation were used for confirmation (SIS 028166, 1982).

Twenty colonies with metallic sheen were isolated from each sample when ever possible. Starting from a selected point, all colonies were isolated until the desired number had been counted. All strains positive in the confirmation

test in the MPN procedure were isolated for identification. The strains were purified by streaking on tryptone yeast extract agar two to three times before identification with API 20 E kits (API Systems S.A., La Balme Les Grottes, 38390 Montalieu Vercieu, France). Storage of strains from about two weeks to one and a half years was necessary before identification. The strains were stored in tubes in a medium containing 8 grams Lab Lemco Broth and 5.5 grams agar (Difco, Bacto) in one litre, sealed airtight, and kept at room temperature in the dark after incubation at 35 °C overnight. The oxidase test was carried out on colonies growing on tryptone yeast extract plates using the Pathotec oxidase test (General Diagnostics) or the reagent of Kovacs (1956). Gram staining was carried out using colonies from the same medium.

2.43 Enumeration and identification of thermotolerant coliform bacteria

In the colony count techniques modified mFC medium containing Water Blue as the indicator (SFS 4088, 1983) was used. The plates were incubated at 44±0.5 °C for 22±2 hours. In the MPN procedure the thermotolerance of those bacteria giving a positive result in the confirmation procedure was tested using EC broth (APHA 1980) and incubation at 44±0.5 °C for 22±2 hours.

Ten typical blue colonies were isolated from mFC plates prepared from each sample when ever possible. All thermotolerant coliform bacteria from the MPN procedure were isolated. The thermotolerant coliform bacteria were identified as described for total coliform bacteria (Section 2.42).

In the experiment on diurnal variation, only the oxidase test and indole production at 44 °C in 24 hours were tested as decribed in the standard SFS 4088 (1983).

2.44 Enumeration and identification of fecal streptococci

KF Streptococcus agar (Difco, lot number 688821) and incubation at 35±1 °C for 44±4 hours (Kenner et al. 1961) were used in the enumeration of fecal streptococci in 1981 and 1982. At a later date, Havelaar et al. (1983) reported that the azide concentration of this agar lot was too low for the complete inhibition of non-streptococci. In the diurnal variation study (Section 3.12) lot number 713801 was used. Its azide concentration and selectivity were found to be satisfactory at the National Institute of Public Health, Bilthoven, the Netherlands. However, no clear differences were noticed in the results obtained with different lots of KF Streptococcus agar. The great majority of observations of fecal streptococci (Section 3.11) were from cultivation on m-Enterococcus agar (SFS 3014, 1984).

In the MPN procedure azide dextrose broth and incubation at 35±1 °C for 44±4 hours were used for primary cultivation (SFS 3015, 1985). In the confirmation BEA (bile esculin azide) agar (Difco) was used and the plates were incubated at 35±1 °C for 22±2 hours. Due to the incubation temperature, this confirmation test is less selective than the one used for colony counting techniques (Engel and Soedirman 1976).

When enough colonies grew on the plates, 30 strains were isolated from influent and effluent samples during the growing season from the Tyyrinvirta farm.

Fig. 3 illustrates the procedures for the characterization and identification of these isolates. The Gram stain was prepared from cells growing on agar and not from broth culture. Therefore *Streptococcus* strains may have given negative Gram reactions and may have appeared rod-like in smears (Deibel and Seeley 1974, Facklam and Wilkinson 1981).

In the experiment on diurnal variation, colonies were isolated from KF Streptococcus agar, purified twice on brain heart infusion (BHI) agar (Difco) and tested for esculin hydrolysis at 44±0.5 °C for 44±4 hours on BEA agar.

2.45 Enumeration of *Aeromonas hydrophila*

The mA medium of Rippey and Cabelli (1979) and incubation at 35±1 °C for 22±2 h were used to enumerate *A. hydrophila* from the influent and effluent water at both fish farms in 1981. The mannitol test was carried out *in situ* as described in the original paper of Rippey and Cabelli, but for the oxidase test usually ten strains from each sample were streaked onto tryptone yeast extract agar before the oxidase test in order to avoid the interference of oxidase reaction caused by growth on the medium containing sugar (Havelaar et al. 1980).

2.5 Statistical analyses and data processing

BMDP statistical software and an Eclipse 6000 computer were used for the simple data description and the correlation analysis (Section 3.11). Before

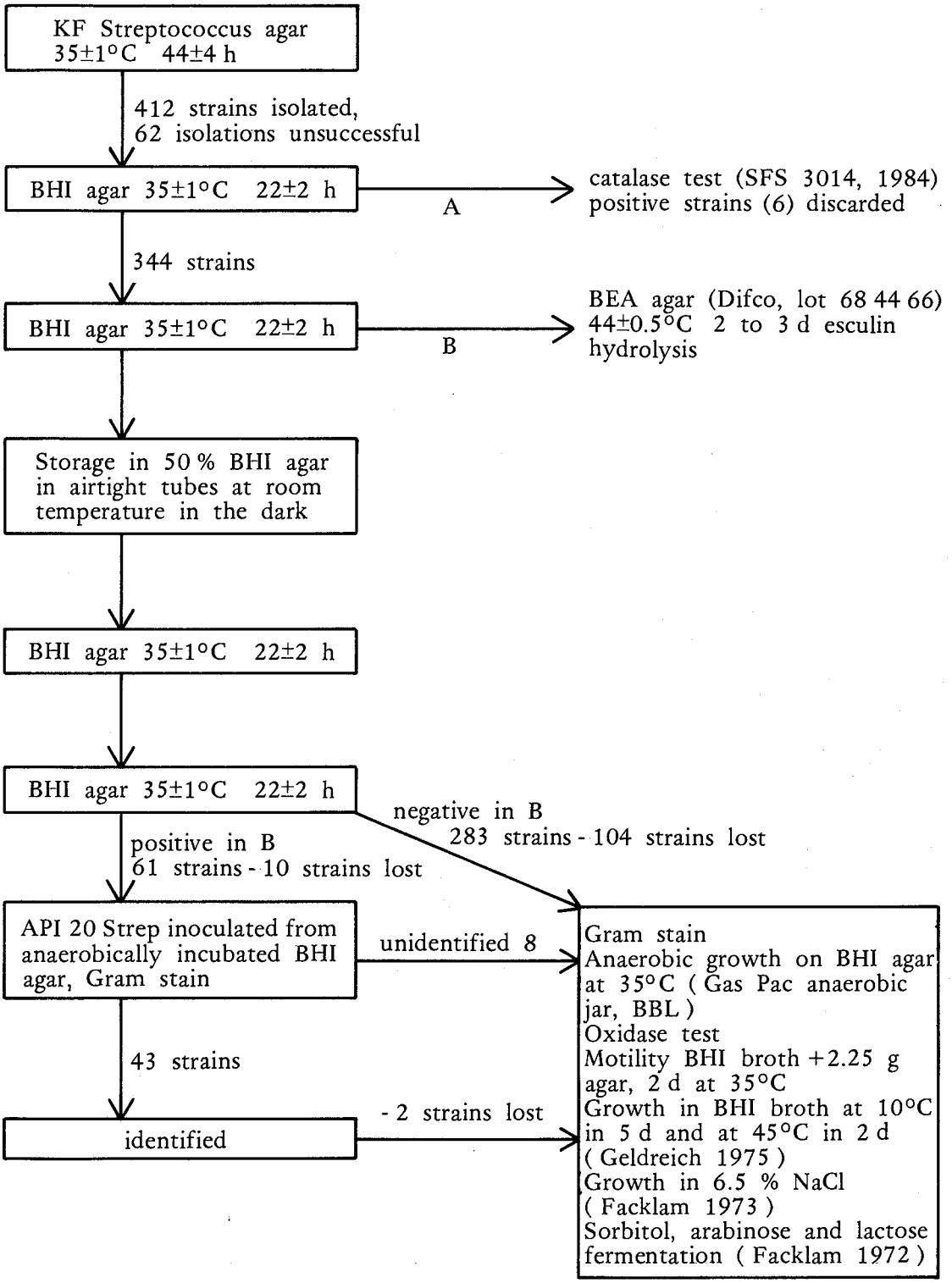

Fig. 3. Characterization of presumptive fecal streptococci from KF Streptococcus agar.

the correlation analysis, the changes in concentrations of bacteria, and of both chemical and physical variables at the fish farms were calcuted. This was done in order to allow the comparison of results from different fish farms using different water qualities. In order to cover a wide range of situations and include negative and zero values, 100 was added to all values of concentrations of bacteria before the logarithmic transformation.

In most cases, the 95 % confidence limits for bacterial counts were calculated according to the method of Niemelä (1977). When the confirmation test was used, the confirmed colony count and the standard error were calculated as follows (Niemelä, personal communication):

$$x = \frac{k}{n} y,$$

where y = number of counted colonies
n = number of isolated strains
k = number of confirmed strains
x = confirmed colony count

$$s(x) = \sqrt{\frac{ky}{n^3}(ny - ky + nk)} \quad ,$$

where $s(x)$ = standard error of the mean

The standard error was multiplied by two to obtain the 95 % confidence limits.

The χ^2-test was calculated according to the method of Mäkinen (1974).

The means weighted with the flow of the effluent fractions were calculated for bacterial, chemical and physical variables at the Tyyrinvirta and Äyskoski farms (Sections 3.11 and 3.13).

Special programmes were designed to sort and merge the API 20 E codes and identification results so that different enumeration methods, sources of samples, and sampling dates could be observed separately. The programmes were run using the Eclipse 6000 computer.

2.6 Numerical taxonomy

The cluster analysis was carried out in 1985 by Dr. J. Schindler (Department of Medical Microbiology, Charles University, Prague). Jaccard's coefficient of similarity and grouping by simple linkage were used.

For the computer identification the Hewlett Packard 85 desk top computer and the programmes available at the Department of Microbiology, University of Helsinki, were used. The matrix was constructed by the NAMAT 2 program using the data of Brenner (1984) and Izard et al. (1981) of the lactose positive *Enterobacteriaceae* species, together with *Hafnia alvei* and *Serratia liquefaciens*, which are observed as coliforms even if they are regarded as lactose negative. The IPRHEL program was used for the identification. The data of Brenner gave only approximate separation of frequencies of characteristics. They were modified in the matrix as follows: + = 95, − = 5, [+] = 83, [−] = 18, and d = 50. In the API 20 E data base more accurate percentages are used. However, the division into taxa is different in these two systems.

3 RESULTS

3.1 Bacterial, chemical and physical water characteristics at fish farms

3.11 Observations at 27 fish farms

The observations on coliform bacteria, fecal streptococci and physical and chemical characteristics in influent and effluent water have been collected (Table 2). The samples from influent and effluent water were taken at about the same time and therefore they do not necessarily represent the same water mass. Depending on the temporal variation of bacterial density and of concentrations of chemical constituents in the influent and effluent water, the lack of temporal coordination may affect the results. This may explain the occasional decreases in bacterial densities and in the concentrations of chemical constituents.

Only the observations between May 1 and October 31 were considered, because no increases in bacterial concentrations were observed during the winter. If the observation period had been more limited, the mean values for increases in concentrations of bacteria would have been higher. The mean increase in the number of bacteria at fish farms was positive for all bacterial groups (Table 2), although individual zero or negative values were observed twenty times for thermotolerant coliform bacteria, four times for total coliform bacteria and twenty-four times for fecal streptococci.

The data covered a large temperature range (Table 2). The increase in the concentration of total nitrogen at fish farms accounted on average for 45 % of the effluent nitrogen. The increase in the concentration of total phosphorus at fish farms

Table 2. Description of the data on bacteriological, chemical and physical water characteristics at 27 fish farms. (FS = fecal streptococci, TC = total coliforms, FC = thermotolerant coliforms, N_{tot} = total nitrogen, P_{tot} = total phosphorus)

Variable, dimension	Total frequency	Mean	Standard deviation	Smallest value	Largest value
1) Increase in FS, CFU in 100 ml	170	3 320	15 780	—96	120 650
2) Increase in TC, CFU in 100 ml	54	680	1 111	—22	4 222
3) Increase in FC, CFU in 100 ml	58	104	613	—85	4 638
Effluent temperature, °C	171	13.6	5.1	0.5	23.4
Increase in N_{tot}, $\mu g\ l^{-1}$ N	179	312	235	—280	1 420
Effluent N_{tot}, $\mu g\ l^{-1}$ N	179	686	245	248	1 700
Increase in P_{tot}, $\mu g\ l^{-1}$ P	179	65	41	2	219
Effluent P_{tot}, $\mu g\ l^{-1}$ P	179	81	43	9	240
Reduction in pH	179	0.3	0.3	—0.3	2.2
Effluent pH	180	6.7	0.2	5.5	7.5
Increase in conductivity, mS m^{-1}	179	0.2	0.3	—0.8	1.1
Effluent conductivity, mS m^{-1}	179	5.2	1.4	2.6	9.9
Increase in BOD_7, mg l^{-1} O_2	57	0.9	1.1	—3.7	3.1
Effluent BOD_7, mg l^{-1} O_2	57	2.3	1.3	0.5	6.2
Reduction in oxygen saturation, %	147	22	12	—7	49
Effluent oxygen saturation, %	161	73	14	37	117
1) lg (increase in FS + 100)	165	2.44	0.69	0.60	5.08
2) lg (increase in TC + 100)	49	2.57	0.53	2.00	3.64
3) lg (increase in FC + 100)	53	2.07	0.30	1.18	3.68

was even more drastic, accounting for 80 % of the effluent phosphorus. The pH was slightly lower and the conductivity slightly higher in the effluent than in the influent. The concentration of biologically degradable matter often increased at fish farms. The reduction in oxygen saturation was on average 22 %, but variations were considerable. The respiration of fish naturally explains an important part of the consumption of dissolved oxygen. The significance of microbial metabolism in excreted fish feces and unconsumed feed was not measured.

Interesting differences were observed in the correlations between different indicator bacteria and chemical and physical water characteristics (Table 3). There was no correlation between increase in thermotolerant coliform bacteria and increase in total coliform bacteria. Thermotolerant coliform bacteria were also uncorrelated with fecal streptococci and with chemical and physical water characteristics. On the other hand, the increases in total coliform bacteria and fecal streptococci were strongly correlate. Both of these bacterial groups were also correlated with many of the physical and chemical water characteristics.

The correlations of the increase coefficient (lg of the effluent concentration minus lg of the influent concentration) of thermotolerant coliform bacteria suggest that phenomena different from those affecting the other bacterial groups were involved.

The chemical and physical variables were frequently intercorrelated (Table 4). The correlations of the variable concentrations in the effluent with the changes of the corresponding concentrations at fish farms imply significant water quality change due to fish farming or the coincidence of water quality change at fish farms with seasonal changes in water bodies.

3.12 Diurnal variation of water quality at the Tyyrinvirta farm

Water temperature decreased slightly during the obervation period (Fig. 4). There were no drastic temporal changes in the chemical or physical variables of the influent or effluent waters during the experiment. However, the effects of fish farming on water quality were consistently similar to the effects observed for the 27 fish farms (Section 3.11): the oxygen saturation decreased (35 %), the conductivity increased (0.4 mS m^{-1}), the pH decreased (0.6), the concentration of total nitrogen increased (420 $\mu g\ l^{-1}$), and the concentration of total phosphorus increased (65 $\mu g\ l^{-1}$). The concentrations of nitrogen and phosphorus were lower in the morning samples of effluents than later in the day.

In the influent water the density of fecal streptococci was on average 22 CFU in 100 ml.

Table 3. The correlation coefficients between bacterial, chemical and physical variables. r = correlation coefficient, d.f. = degrees of freedom. Significant at the * = 95 %, ** = 99 %, *** = 99.9 % confidence level. Abbreviations as in Table 2.

Variable	Increase in FS		Increase in TC		Increase in FC		Increase coefficient of FS		Increase coefficient of TC		Increase coefficient of FC	
	r	d.f.	r	d.f.	r	d.f.	r	d.f.	r	d.f.	r	d.f.
Increase in TC	0.668***	45										
Increase in FC	—0.277	39	0.108	28								
Effluent temperature	0.243**	151	0.292*	48	—0.060	51	0.185*	152	0.142	45	—0.053	52
Increase in N_{tot}	0.079	163	0.362**	47	0.020	51	0.213**	164	0.206	44	0.102	52
Effluent N_{tot}	0.101	163	0.386*	47	0.131	51	0.187*	164	0.219	44	0.055	52
Increase in P_{tot}	0.066	163	0.340*	48	0.113	52	0.183*	164	0.275	45	0.402**	53
Effluent P_{tot}	0.031	163	0.289*	48	0.149	52	0.165*	164	0.234	45	0.406**	53
Reduction in pH	0.428***	163	0.288*	48	—0.032	52	0.289***	164	0.126	45	0.035	53
Effluent pH	—0.255**	165	—0.330*	48	0.090	52	—0.216**	165	—0.146	45	0.275*	53
Increase in conductivity	0.088	163	0.160	48	0.189	52	0.178*	164	0.143	45	0.278*	53
Effluent conductivity	—0.157*	163	—0.541*	48	0.335*	52	—0.134	164	—0.319*	45	0.317*	53
Increase in BOD_7	0.266	45	0.837	2	0.010	12	0.120	46	0.532	2	0.125	12
Effluent BOD_7	0.450**	45	0.561	2	0.119	12	0.349*	46	0.817	2	0.215	12
Reduction in oxygen saturation	0.335***	133	0.335*	44	0.115	51	0.382***	134	0.152	43	0.269	50
Effluent oxygen saturation	—0.328***	147	—0.510***	46	—0.015	51	—0.294***	148	—0.234	43	0.000	53
Increase coefficient of												
FS	0.737***	163	0.569***	45	—0.160	41						
TC	0.230	42	0.626***	45	0.201	27	0.218	42				
FC	—0.247	42	0.154	29	0.797***	49	—0.037	41	0.389*	26		

Table 4. Correlation coefficients between chemical and physical variables. Degrees of freedom in brackets below the corresponding correlation coefficient. Significant at the * = 95 %, ** = 99 % and *** = 99.9 % confidence level. Abbreviations as in Table 2.

Variable	Effluent temperature	Increase in N_{tot}	Effluent N_{tot}	Increase in P_{tot}	Effluent P_{tot}	Reduction in pH	Effluent pH	Increase in conductivity	Effluent conductivity	Increase in BOD_7	Effluent BOD_7	Reduction in oxygen saturation
Increase in N_{tot}	0.074											
	(166)											
Effluent N_{tot}	0.087	0.887***										
	(165)	(178)										
Increase in P_{tot}	0.085	0.675***	0.592***									
	(165)	(177)	(177)									
Effluent P_{tot}	0.107	0.666***	0.611***	0.974***								
	(156)	(177)	(177)	(178)								
Reduction in pH	0.209**	0.247***	0.267***	0.463***	0.470***							
	(166)	(177)	(177)	(177)	(177)							
Effluent pH	—0.128	—0.247***	—0.359***	—0.208**	—0.201**	0.037						
	(166)	(178)	(178)	(178)	(178)	(178)						
Increase in conductivity	0.122	0.435***	0.333***	0.486***	0.471***	0.178*	—0.256***					
	(166)	(177)	(177)	(177)	(177)	(178)	(178)					
Effluent conductivity	—0.175*	—0.216**	—0.258***	—0.137	—0.109	—0.083	0.252***	—0.022				
	(165)	(177)	(177)	(177)	(177)	(177)	(178)	(177)				
Increase in BOD_7	0.359**	0.471***	0.468***	0.463***	0.346**	0.514***	—0.134	0.273*	0.010			
	(56)	(56)	(56)	(56)	(56)	(56)	(56)	(56)	(55)			
Effluent BOD_7	0.447***	0.552***	0.592***	0.649***	0.679***	0.637***	—0.152	0.365**	—0.014	0.666***		
	(56)	(56)	(56)	(56)	(56)	(56)	(56)	(56)	(55)	(56)		
Reduction in oxygen saturation	0.473***	0.462***	0.436***	0.603***	0.598***	0.545***	—0.279***	0.444***	—0.189*	0.310*	0.514***	
	(144)	(145)	(145)	(145)	(146)	(146)	(146)	(146)	(145)	(54)	(54)	
Effluent oxygen saturation	0.077	—0.412***	—0.408***	—0.541***	0.537***	—0.324***	0.217**	—0.258***	0.097	0.005	—0.135	—0.605***
	(158)	(159)	(159)	(159)	(159)	(160)	(160)	(160)	(159)	(54)	(54)	(146)

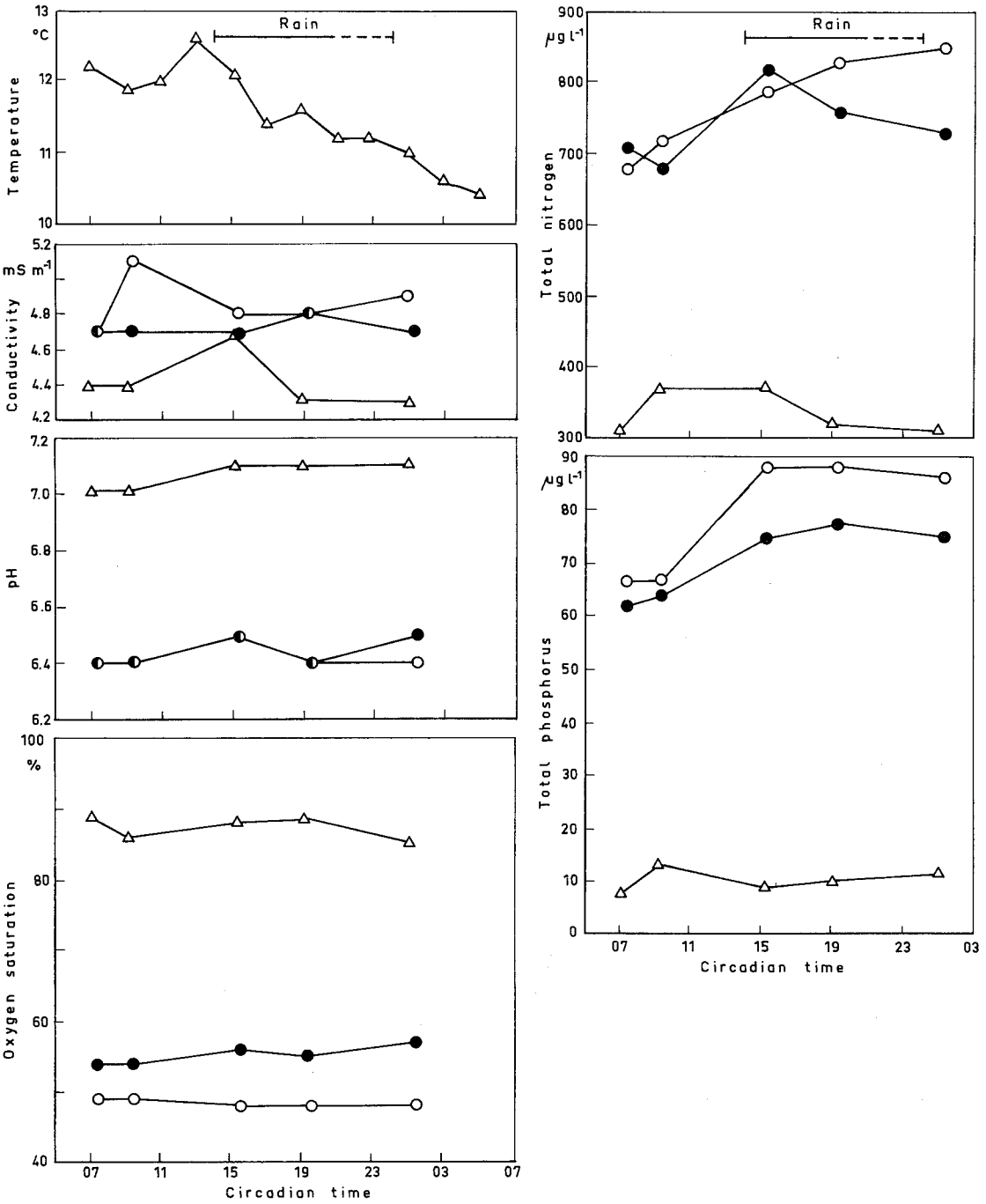

Fig. 4. Diurnal variation of physical and chemical water characteristics at the Tyyrinvirta farm on the 4th to 5th of September 1984.

△ - - △ Influent
○ - - ○ Rearing channel effluent
● - - ● Middle channel effluent

Their density increased from 17 h to 03 h, the peak value being 65 CFU per 100 ml at 23 h (Fig. 5). In the effluent fractions the average density was 88 times the influent density. The morphology of colonies growing on KF Streptococcus agar from fish farm effluents was variable. Minute pin-point colonies were frequently observed. The difficulties in counting colonies explain to some extent the variation in density of fecal streptococci, but part of the variation probably was temporal variation.

In the effluent there was a maximum temporal variation of about fivefold in the concentration of presumptive fecal streptococci. The peak values were not observed at the same time in the rearing channel effluent and in the middle channel effluent. The feeding, and the fish movement which it causes, did not affect the whole farm at the same time. The detention time of water in different parts of the complex pond and raceway system varies and, therefore, the theoretical detention time for the whole farm (3.6 hours during the experiment) could not be used and the variation in water quality parameters was not in phase in the two effluent fractions. However, the concentration of these bacteria was lower at night than during working hours.

The counting of colonies of total coliform bacteria was not satisfactory (Section 2.42) and therefore the error in the results was relatively large. In the influent water the density of total coliform bacteria was on average 29 CFU per 100 ml. In the effluents their density was much higher (Fig. 6).

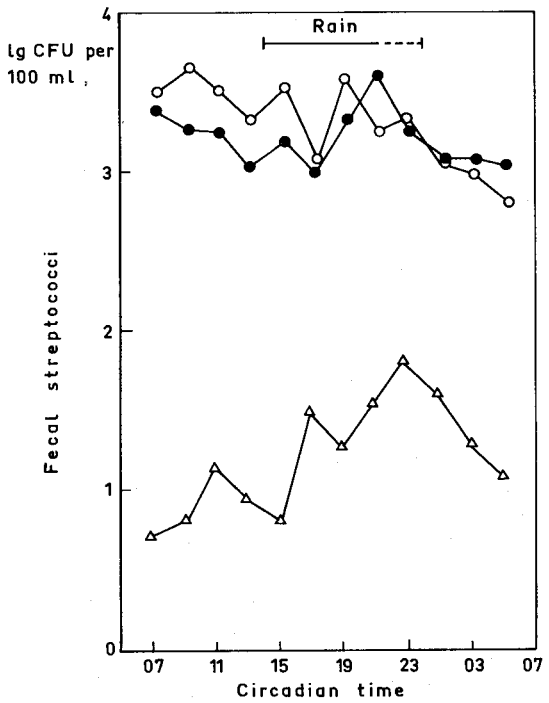

Fig. 5. Diurnal variation of concentration of presumptive fecal streptococci at the Tyyrinvirta farm on the 4th to 5th of September 1984. (Averages of duplicate samples. CFU = colony forming unit.)
Δ - - Δ Influent
○ - - ○ Rearing channel effluent
● - - ● Middle channel effluent

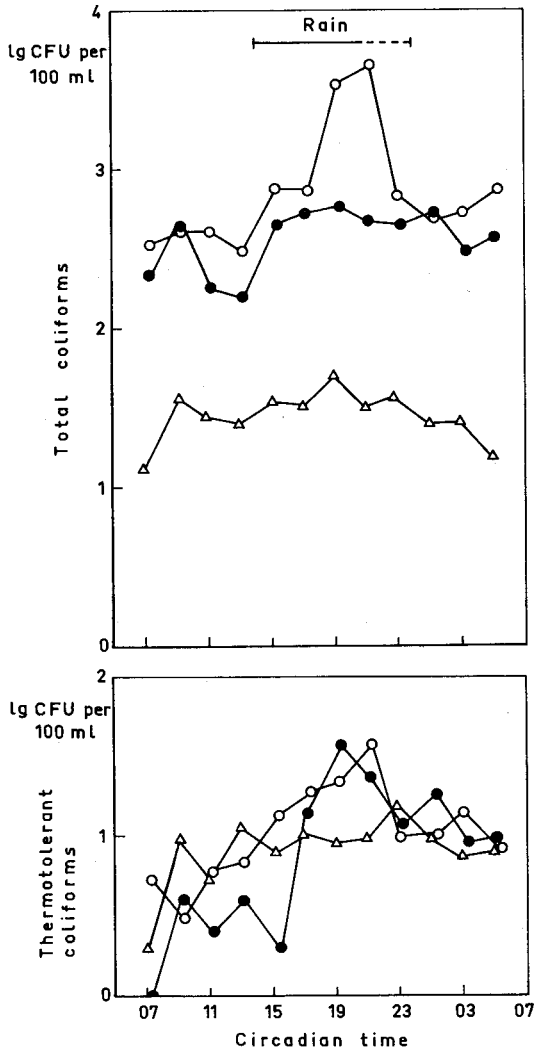

Fig. 6. Diurnal variation of concentration of coliform bacteria at the Tyyrinvirta farm on the 4th to 5th of September 1984. (Averages of duplicate samples. CFU = colony forming unit.)
Δ - - Δ Influent
○ - - ○ Rearing channel effluent
● - - ● Middle channel effluent

In the effluent water the temporal variation of total coliform bacteria was not as great as in the case of presumptive fecal streptococci, except during the rainfall, when the concentration of total coliform bacteria in the rearing channel effluent increased markedly. This increase coincided with the slight increase in thermotolerant coliform bacteria at the farm.

In the influent water the density of thermotolerant coliform bacteria was on average nine CFU per 100 ml, the range being from two to 16 CFU per 100 ml. In the effluent waters the densities of these bacteria were at the same level as in the influent water except during the rainfall when they reached peak values (Fig. 6).

A total of 140 strains of thermotolerant coliform bacteria were isolated and tested for oxidase production and indole production at 44 °C after purification on tryptone yeast extract agar (Table 5). The isolations were performed from samples taken between 19 and 21 h, when the peak densities were observed. All the strains were oxidase negative. It can be concluded that the vast majority of thermotolerant coliform bacteria both in the influent and effluent waters were presumptive *E. coli.* Since no higher colony counts of thermotolerant coliform bacteria were observed in the influent before 19 h, the comparison of the 19 and 21 h results of the influent and the effluents fractions was concerned acceptable. The numbers of presumptive *E. coli* with their 95 % confidence limits at 19 h were in the influent 9±4, in the middle channel effluent 21±7, in the rearing channel effluent 36±9, and at 21 h in the influent 9±4, in the middle channel effluent 34±9, and in the rearing channel effluent 15±7 CFU per 100 ml. The slight increase or presumptive *E. coli* seems to occur during the rainfall.

Table 5. Indole production by thermotolerant coliform bacteria isolated from fish farm waters at diurnal peak density (Fig. 6, 19 and 21 hours).

Sample	No. of strains		Indole positive	
	isolated	lost	no. of strains	%
Influent	33	0	31	94
Rearing channel effluent	58	0	54	93
Middle channel effluent	49	0	41	84
Total effluent	107	0	95	89

Table 6. Esculin hydrolysis on BEA agar at 44 °C by fecal streptococci isolated from fish farm waters during the diurnal variation experiment.

Sample	Hour	No. of strains		Esculin positive	
		isolated	lost	no. of strains	%
Influent	07	10	1	6	
	09	12	0	8	
	19	14	0	8	
	21	32	1	33	
	total	68	2	49	72
Rearing channel effluent	07	30	0	0	
	09	30	0	0	
	19	30	1	3	
	21	30	0	2	
	total	120	1	5	4.2
Middle channel effluent	07	30	2	0	
	09	30	1	0	
	19	30	1	0	
	21	30	0	0	
	total	120	4	0	0
Total effluent		240	5	5	2.1

Altogether 308 strains of presumptive fecal streptococci were isolated and tested for esculin hydrolysis on BEA agar (Table 6). The majority of the influent strains gave a positive reaction in this confirmation test, whereas only a few of the effluent strains were positive.

The peak values of presumptive fecal streptococci were observed in the effluents after working hours of the farm (Fig. 5). The concentrations of the total coliform bacteria and thermotolerant coliform bacteria were high in the evening (Fig. 6) and, furthermore, the only confirmed fecal streptococci from effluent water were isolated from the evening samples.

3.13 Variation of water quality at the Tyyrinvirta and Äyskoski farms during the growing season

Water temperature was similar in 1981 and 1982 at the Tyyrinvirta farm (Fig. 7). At the Äyskoski farm, further upstream in the watercourse, the maximum water temperature was slightly lower than at the Tyyrinvirta farm in 1981. In 1981 the maximum volume of water was used at the farms when the water temperature was high. Because of increased fish biomass, the flow was kept higher at the end of summer than in the early summer in spite of reduction in water temperature. Although the water flow was increased during the period of high water temperature the greatest reductions in oxygen saturation were observed at the farms during the same period. There was a tendency to two maxima during the growing season in water flow, reduction in oxygen saturation (change between influent and effluent concentrations) and

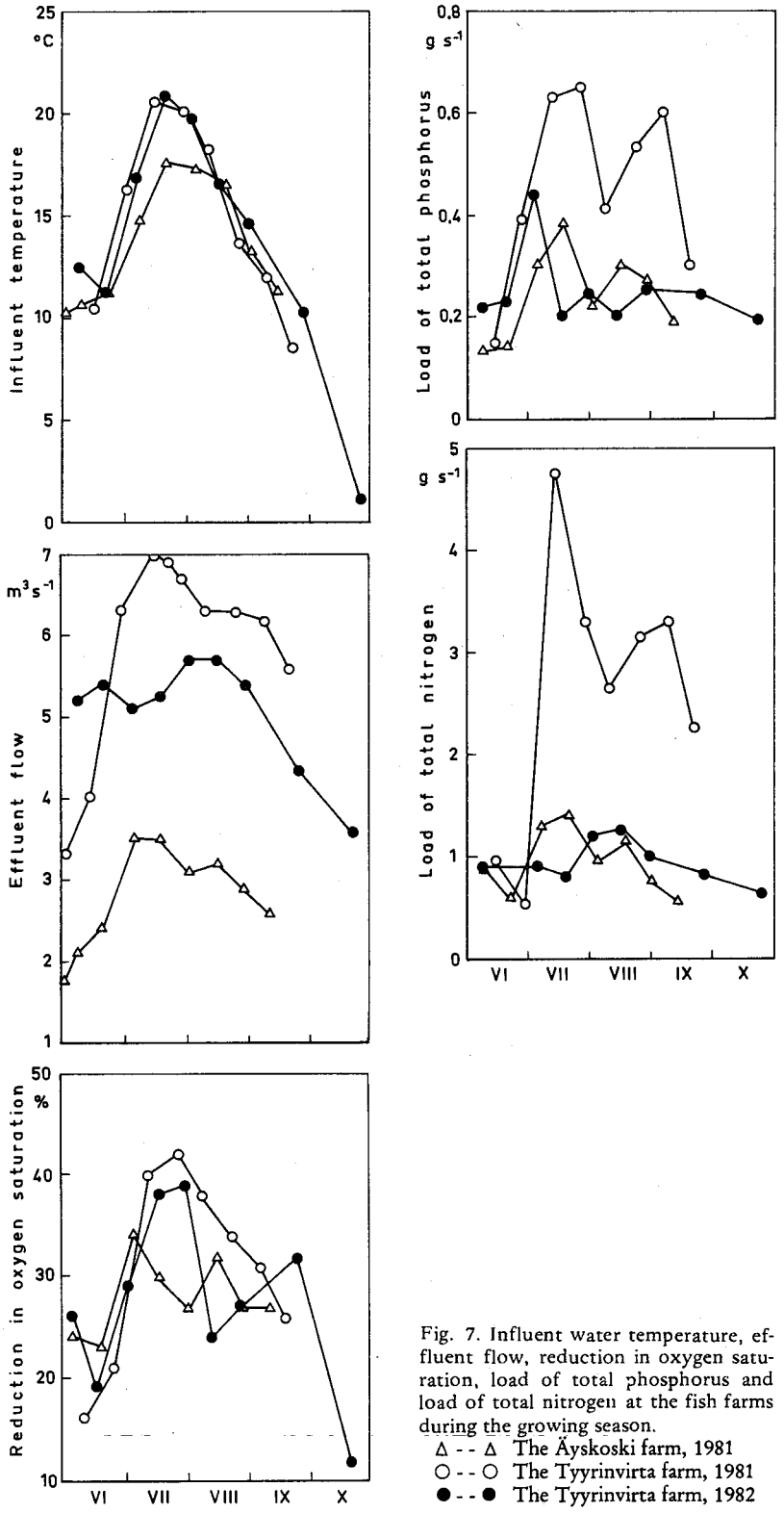

Fig. 7. Influent water temperature, effluent flow, reduction in oxygen saturation, load of total phosphorus and load of total nitrogen at the fish farms during the growing season.

△ - - △ The Äyskoski farm, 1981
○ - - ○ The Tyyrinvirta farm, 1981
● - - ● The Tyyrinvirta farm, 1982

in the amounts of phosphorus and nitrogen discharged to the watercourse.

The concentration of phosphorus in the influent at the farms was about 10 $\mu g\ l^{-1}$, whereas the average concentration in the effluents was 7.5 times higher (Appendices 1 to 3). Of the total phosphorus increase, 56 % was in the form of phosphate. The nitrogen concentrations increased at the farms so that the effluent concentrations were from 1.5 to 2 times higher than the influent concentrations. Chemical oxygen demand and conductivity increased and pH decreased slightly but consistently at the farms.

The concentrations of presumptive fecal streptococci varied in the influent water, but they were always lower than in the effluent (Tables 7 to 9). The sharp increase of fecal streptococci at the farms started in June, simultaneously with the increase in temperature, and the remarkable increase continued to the autumn (Fig. 8). The

Table 7. The concentrations of bacteria in water at Tyyrinvirta in 1981. CFU in 100 ml ± 95 % confidence limit; I = influent, M = middle channel effluent, R = rearing channel effluent. Averages of duplicate samples.

Date	Sample	CFU per 100 ml ± 95 % confidence limits			
		Fecal streptococci	Total coliforms	Thermotolerant coliforms	Aeromonas hydrophila
June 2nd	I	0	8 ± 6	0	
	M	2 ± 3	16 ± 8	1 ± 2	
	R	5 ± 5	120 ± 69	1 ± 2	
June 15th	I	46 ± 10	280 ± 75	19 ± 6	
	M	150 ± 54	260 ± 72	20 ± 6	
	R	100 ± 45	510 ± 100	37 ± 9	
June 23rd	I		20 ± 28	1 ± 2	
	M		46 ± 41	6 ± 5	
	R		100 ± 60	8 ± 6	
June 29th	I	(37 ± 9)	14 ± 5	8 ± 4	0
	M	640 ± 110	(250 ± 220)	4 ± 3	1 800 ± 1 000
	R	430 ± 92	185 ± 61	11 ± 5	2 800 ± 1 500
July 9th	I		9 ± 6	3 ± 3	
	M		320 ± 110		
	R		150 ± 77	10 ± 20	
July 14th	I	30 ± 8	32 ± 8	13 ± 5	
	M	3 000 ± 780	470 ± 92	28 ± 7	
	R	8 100 ± 1 300	1 100 ± 150	15 ± 5	
July 21st	I	530 ± 150	62 ± 16	33 ± 11	
	M	7 800 ± 1 800	200 ± 89	25 ± 10	
	R	11 500 ± 1 200	720 ± 160	50 ± 14	
July 28th	I	77 ± 12	150 ± 60	73 ± 12	300 ± 100
	M	6 400 ± 1 100	950 ± 130	93 ± 14	31 000 ± 7 800
	R	28 500 ± 7 600	2 250 ± 670	110 ± 14	21 000 ±7 600
August 10th	I	48 ± 10	64 ± 11	49 ± 9	90 ± 40
	M	7 050 ± 1 200	1 600 ± 540	27 ± 7	3 100 ± 400
	R	7 400 ± 1 200	1 200 ± 460	66 ± 11	4 900 ± 700
August 25th	I	23 ± 7	27 ± 7	0	110 ± 66
	M	1 700 ± 170	1 200 ± 460	0	20 000 ± 6 400
	R	3 000 ± 770	360 ± 260	0	2 500 ± 1 400
September 9th	I	38 ± 12	37 ± 12	6 ± 5	(50)
	M	6 700 ± 1 600	300 ± 110	5 ± 4	1 200 ± 520
	R	17 200 ± 2 600	180 ± 81	3 ± 3	600 ± 360
September 22nd	I	36 ± 12	7 ± 5	7 ± 5	6 ± 12
	M	14 300 ± 2 400	250 ± 94	11 ± 7	490 ± 340
	R	5 500 ± 1 400	230 ± 96	11 ± 7	390 ± 280

proliferation of these bacteria at the farms did not appear to follow accurately the changes in chemical water characteristics.

In the influent, total coliform bacteria were present in higher concentrations at Tyyrinvirta than at Äyskoski. However, only one sample at Äyskoski was negative. The numbers of total coliform bacteria and presumptive fecal streptococci tended to be elevated at the same time in the influent waters (Tables 7 to 9). The increase in total coliform bacteria was appreciable (Fig. 9), but not quite as great as the increase in presumptive fecal streptococci (Fig. 8). The increase started when water temperature increased. However, there was a tendency towards two maxima in the proliferation of total coliform bacteria, which was not observed in water temperature but was noticeable in the effluent volume (Figs. 7 and 9). No direct relationship was observed between the increase in total coliform bacteria on the other

Table 8. The concentrations of bacteria in water at Tyyrinvirta in 1982. Abbreviations as in Table 7. Averages of duplicate samples.

Date	Sample	CFU per 100 ml ± 95 % confidence limits		
		Fecal streptococci	Total coliforms	Thermo-tolerant coliforms
June 8th	I	20 ± 6	8 ± 4	4 ± 3
	M	750 ± 390	22 ± 7	8 ± 4
	R	(59 ± 10)	29 ± 5	3 ± 2
June 21st	I	5 ± 3	7 ± 4	2 ± 2
	M	185 ± 55	6 ± 3	2 ± 2
	R	4 200 ± 920	89 ± 13	3 ± 2
July 5th	I	11 ± 5	17 ± 6	6 ± 3
	M	4 550 ± 950	300 ± 77	15 ± 5
	R	>2 600 ± 720		11 ± 5
July 19th	I	23 ± 7	38 ± 9	22 ± 7
	M	21 000 ± 6 400	175 ± 59	19 ± 6
	R	15 000 ± 5 600	1 300 ± 510	36 ± 8
August 2nd	I	120 ± 15	190 ± 62	140 ± 17
	M	46 000 ± 9 600	430 ± 93	48 ± 10
	R	26 000 ± 7 200	550 ± 140	73 ± 12
August 16th	I	17 ± 6	120 ± 49	20 ± 6
	M	165 000 ± 17 000	950 ± 440	47 ± 10
	R	32 000 ± 8 000	3 400 ± 830	23 ± 7
August 30th	I	19 ± 6	190 ± 62	6 ± 3
	M	11 400 ± 1 400	360 ± 84	12 ± 5
	R	7 050 ± 1 200	410 ± 90	12 ± 5
September 27th	I	7 ± 4	6 ± 3	7 ± 4
	M	750 ± 390	110 ± 47	8 ± 4
	R	700 ± 370	260 ± 72	7 ± 4
October 10th	I	10 ± 4	450 ± 95	2 ± 2
	M	150 ± 55	31 ± 10	2 ± 2
	R	80 ± 40	500 ± 99	4 ± 3

Table 9. The concentrations of bacteria in water at Äyskoski in 1981. CFU in 100 ml ±95 % confidence limit; I = influent, S = sedimentation pond effluent, D = direct flow effluent. Averages of duplicate samples.

Date	Sample	CFU per 100 ml ± 95 % confidence limits			
		Fecal streptococci	Total coliforms	Thermotolerant coliforms	Aeromonas hydrophila
June 2nd	I	1 ± 2	1 ± 2	0	
	S	3 ± 3	140 ± 70	1 ± 2	
	D	5 ± 4	130 ± 72	0	
June 9th	I	7 ± 4	0	0	
	S	260 ± 72	160 ± 56	3 ± 2	
	D	1 400 ± 170	270 ± 73	1 ± 1	
June 22nd	I	19 ± 6	16 ± 6	5 ± 3	(50)
	S	65 ± 36	140 ± 53	8 ± 4	(1 300)
	D	70 ± 37	70 ± 37	11 ± 5	(1 300)
June 30th	I	0	4 ± 4	2 ± 3	
	S	>1 000	9 800 ± 200	70 ± 53	
	D	>1 000	9 500 ± 190	110 ± 66	
July 7th	I		4 ± 3	1 ± 1	
	S		2 850 ± 750	4 ± 3	
	D		3 000 ± 770	2 ± 2	
July 13th	I		3 ± 3	1 ± 2	
	S		2 900 ± 1 100		
	D		4 700 ± 1 400		
July 20th	I	(5 ± 3)	2 ± 2	0	
	S	4 600 ± 960	2 850 ± 750	3 ± 2	
	D	1 800 ± 180	4 200 ± 880	2 ± 2	
August 4th	I	5 ± 3	2 ± 2	1 ± 1	70 ± 40
	S	4 600 ± 960	770 ± 370	2 ± 2	2 800 ± 900
	D	1 800 ± 180	1 500 ± 510	0	2 200 ± 780
August 18th	I	36 ± 12	4 ± 3	0	24 ± 30
	S	34 500 ± 8 300	1 800 ± 600	0	8 000 ± 4 000
	D	32 000 ± 8 300	1 950 ± 620	0	8 000 ± 4 000
September 1st	I	21 ± 9	340 ± 117	1 ± 2	27 ± 30
	S	91 000 ± 19 000	4 500 ± 1 300	4 ± 4	2 300 ± 680
	D	160 000 ± 25 000	3 700 ± 1 200	1 ± 2	2 000 ± 620
September 14th	I	110 ± 21	60 ± 49	1 ± 2	(15)
	S	120 000 ± 70 000	3 700 ± 1 200	2 ± 3	630 ± 480
	D	91 000 ± 19 000	3 600 ± 1 200	0	1 700 ± 860

hand and the reduction in oxygen saturation, the increase in total phosphorus and the increase in total nitrogen on the other.

Thermotolerant coliform bacteria were often but not always observed in the influent waters (Tables 7 to 9, Fig. 10). In 1981, elevated concentrations were observed in the influents of both fish farms in June and at Tyyrinvirta at the end of July. In 1982 a peak was not observed in June, but significantly elevated concentrations were noticed in July and August. An important observation on thermotolerant coliform bacteria was that when they occurred in the effluents in higher concentrations than usual, they were as a rule also present in elevated concentrations in the influent (Fig. 10). These bacteria were present in higher concentrations at Tyyrinvirta that at Äyskoski.

The increase coefficients for presumptive fecal streptococci started to increase in June (Fig. 11). Between July and October they were usually between two and four. The increase coefficients of total coliform bacteria were usually lower than those of fecal streptococci, but were however, positive. Higher values were observed at the Äyskoski farm than at Tyyrinvirta. The increase coefficients of thermotolerant coliform bacteria were with one exception low, and negative values were also recorded.

The few measurements of *A. hydrophila* in waters at the fish farms showed that these bacteria were always present in the influent and even in much higher concentrations in the effluent (Tables 7 and 9).

In order to determine the sources of bacteria at fish farms, bacteria were analysed, in addition to

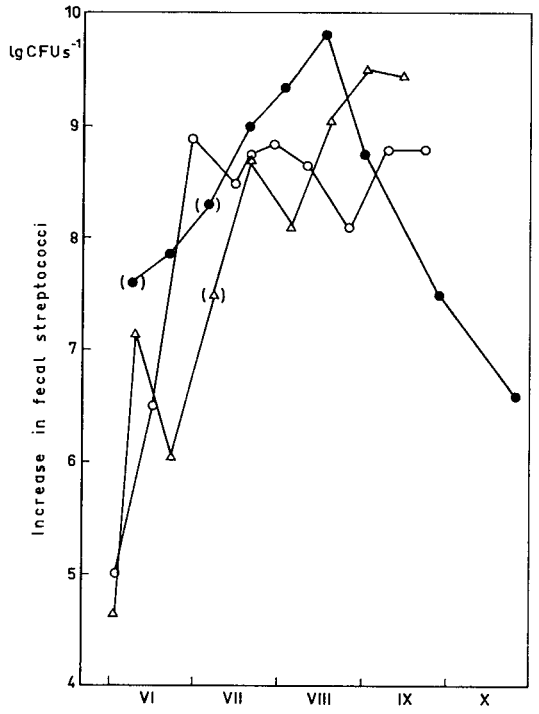

Fig. 8. Increase in presumptive fecal streptococci at fish farms during the growing season. (lg (averages of duplicate samples of effluent fractions weighted with flow — averages of duplicate influent samples) multiplied by flow. CFU = colony forming unit. Brackets indicate result from one effluent fraction or too crowded sample.)
Δ - - Δ The Äyskoski farm, 1981
○ - - ○ The Tyyrinvirta farm, 1981
● - - ● The Tyyrinvirta farm, 1982

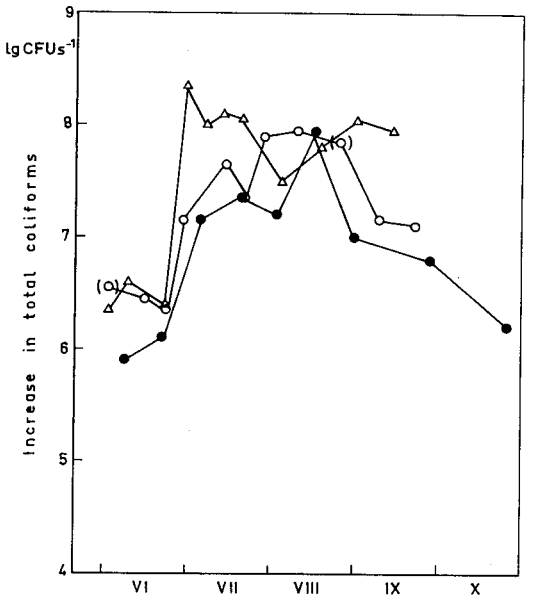

Fig. 9. Increase in total coliform bacteria at fish farms during the growing season. (Description as in Fig. 8)
Δ - - Δ The Äyskoski farm, 1981
○ - - ○ The Tyyrinvirta farm, 1981
● - - ● The Tyyrinvirta farm, 1982

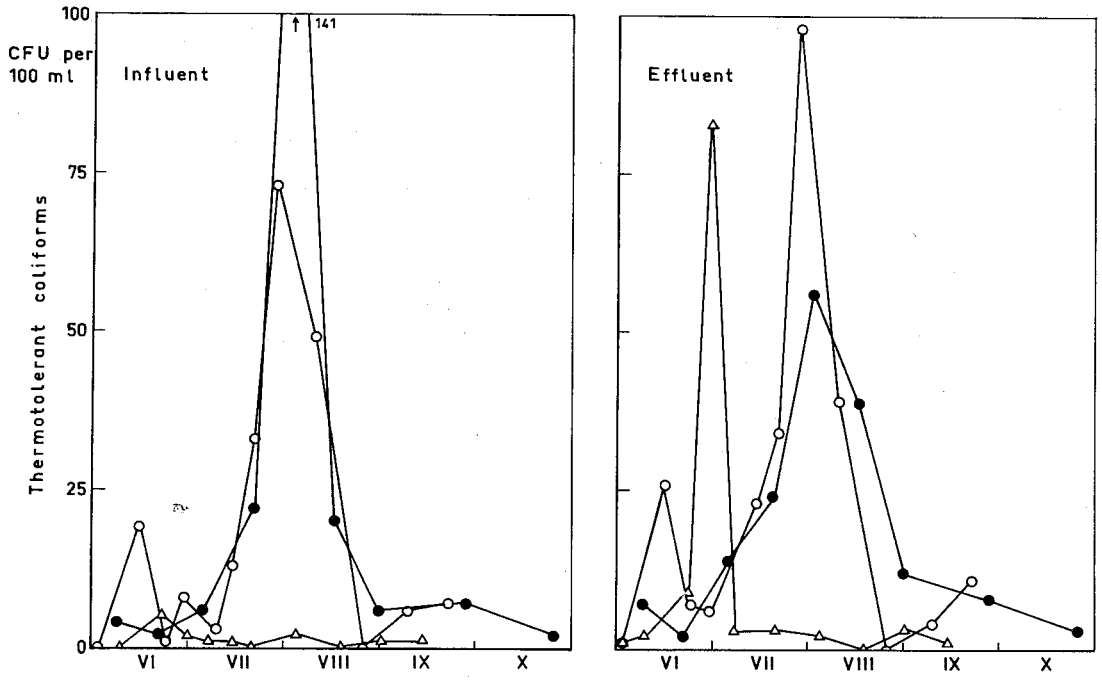

Fig. 10. Occurrence of thermotolerant coliform bacteria in influent and effluent water at the fish farms during the growing season. (Averages of duplicate samples, weighted with the flow of the effluent fractions. CFU = colony forming unit).

Δ - - Δ The Äyskoski farm, 1981
○ - - ○ The Tyyrinvirta farm, 1981
● - - ● The Tyyrinvirta farm, 1982

Table 10. The range of concentrations of bacteria in 15 samples of pelleted fish feed.

Bacterial group	No. of samples in each category (CFU g^{-1})			Maximum
	<0.1	0.1 – 1.0	>1	(CFU g^{-1})
Fecal streptococci[1]	1	5	9	18
Total coliforms	3	7	5	3.4
Thermotolerant coliforms	12	3	0	0.6

[1] confirmed MPN estimates (the selection in this confirmation test is not as efficient as in the confirmation test of colony counting methods)

Table 11. The range of concentrations of bacteria in fish feces (15 samples) and sediment (g^{-1} wet weight, 16 samples) from the bottom of raceway or pool.

	No. of samples in each category (CFU g^{-1})			Maximum
	<10	10—1000	>1000	lg(CFU g^{-1})
Feces				
Fecal streptococci[1]	2	1	12	6.8
Total coliforms[2]	2	3	8	6.0
Thermotolerant coliforms	13	2	0	(1.6)[3]
Sediment				
Fecal streptococci[1]	2	5	9	(6.4)
Total coliforms	0	6	10	(4.2)
Thermotolerant coliforms	14	2	0	1.8

[1] presumptive fecal streptococci counted from pour plates
[2] results from two samples disregarded due to background growth
[3] brackets indicate large difference between duplicate samples or inconsistent dilutions

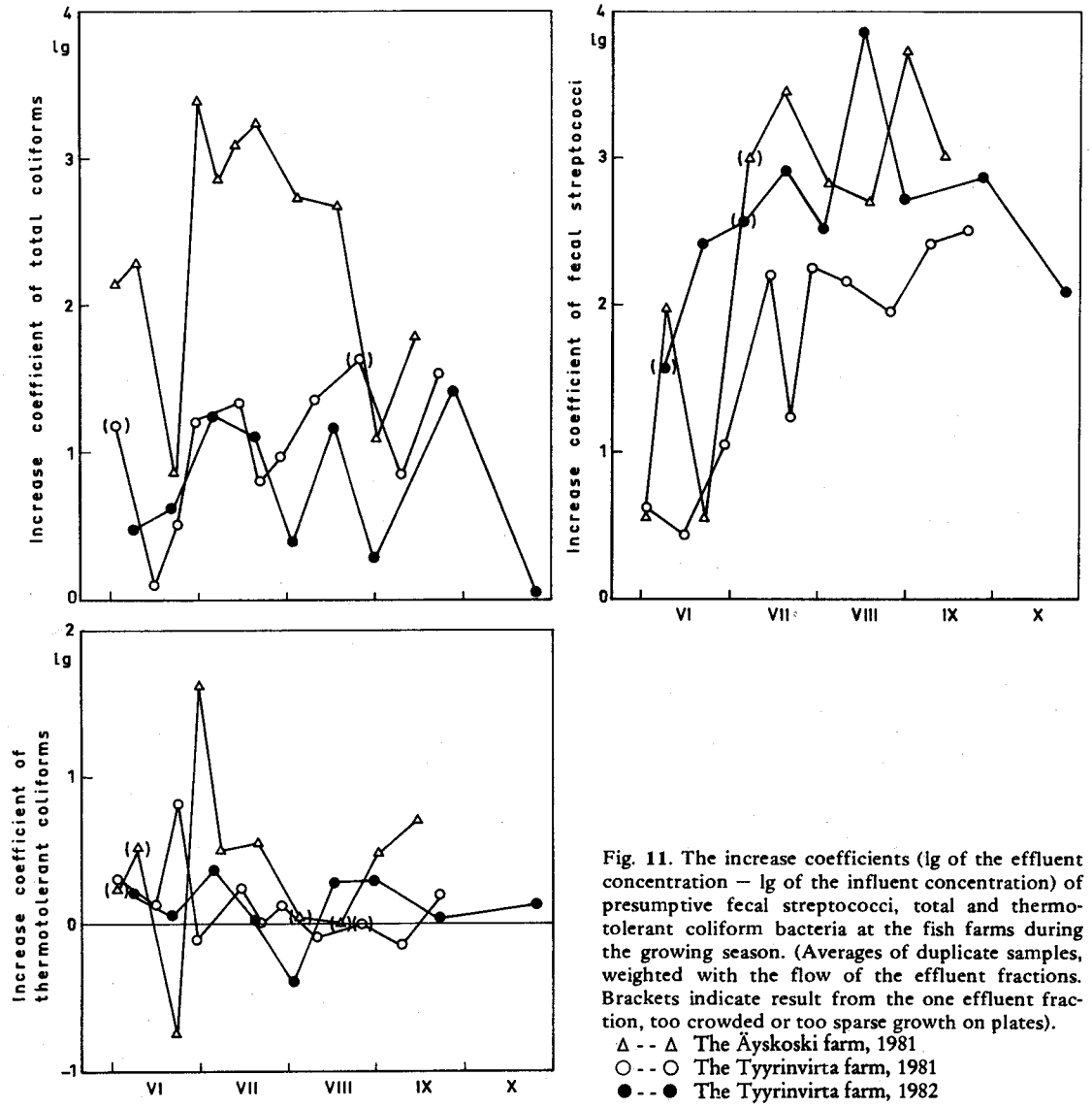

Fig. 11. The increase coefficients (lg of the effluent concentration − lg of the influent concentration) of presumptive fecal streptococci, total and thermotolerant coliform bacteria at the fish farms during the growing season. (Averages of duplicate samples, weighted with the flow of the effluent fractions. Brackets indicate result from the one effluent fraction, too crowded or too sparse growth on plates).
Δ - - Δ The Äyskoski farm, 1981
○ - - ○ The Tyyrinvirta farm, 1981
● - - ● The Tyyrinvirta farm, 1982

water samples, from fish feed, fish feces and sediments. Pelleted fish feed was contaminated sufficiently to produce an inoculum of fecal streptococci and total coliform bacteria, but only occasionally of thermotolerant coliform bacteria (Table 10). These numbers cannot, however, explain the increase of bacteria at the farms. During the study, one sample of ground small fish, used as feed, was taken. It contained 10^5 CFU of total coliform bacteria, from 10^1 to 10^2 CFU of thermotolerant coliform bacteria and 10^3 CFU of fecal streptococci per gram fresh feed. Although the fresh feed was contaminated, its limited use at only one of the farms cannot explain the level of contamination of the effluent waters at the other farm.

The multiplication site for presumptive fecal streptococci and total coliform bacteria was the alimentary tract of fish and probably also the sediment where a fraction of the feces and unconsumed feed deposited (Table 11).

3.14 Evaluation

The low correlation coefficients between the increase of thermotolerant coliform bacteria on the one hand and changes in other indicator bacteria

and chemical and physical variables on the other suggest that the occurrence of thermotolerant coliform bacteria is caused by other phenomena than those causing the increase of other bacterial groups and changes in water quality characteristics (Section 3.11). The different correlations of the increase coefficients of bacterial groups support this impression.

In the waters of fish farms the correlations between bacterial variables and physical and chemical variables appear to imply that fecal streptococci and total coliform bacteria multiply at fish farms concurrently with the water quality changes indicative of general microbial activity, e.g. reduction in pH, increase in conductivity and reduction in oxygen saturation (Section 3.11). However, these correlations are weak and the metabolism of fish and the amount of feed affects the variables strongly.

No drastic diurnal changes were observed in the chemical and physical variables at the Tyyrinvirta fish farm, either in the influent or in the effluent (Section 3.12). However, total phosphorus and total nitrogen concentrations were higher after midday than in the morning. This could have resulted from the feeding, from fish activity (Tervet 1981), or from washout form earth banks at the farm during the rainfall.

Significant increases in concentrations of total coliform bacteria and presumptive fecal streptococci were observed during the diurnal variation experiment at the farm (Section 3.12). The confirmation of the results of primary cultivation of fecal streptococci revealed a clear difference between the strains isolated from the influent and the effluent. In the effluent the confirmed strains were rare (Table 6).

The concentrations of thermotolerant coliform bacteria and presumptive *E. coli* did not increase at the same time as those of fecal streptococci and total coliform bacteria (Figs. 5 and 6, Table 5). However, thermotolerant coliform bacteria and presumptive *E. coli* increased slightly during the rainfall. At the meteorological station at Rastu, 10 km from the farm, a rainfall of 11 mm was measured on the 4th of September. It is highly probable that the detention time of runoff from the shores of Lake Miekkavesi was long enough to prevent elevated concentrations from being observed in the influent at Tyyrinvirta, while runoff from the earth banks at the farm could affect effluent quality soon after the onset of rain.

A large flock of crows (*Corvus corone*) associated with the farm could explain fecal contamination of the banks. The daily loads of thermotolerant coliforms from different birds are known to be considerable, from 10^7 to 10^{11} CFU per bird (Gould and Fletcher 1978, Weiner et al. 1979b, Palmer 1983). *E. coli* is the dominant *Enterobacteriaceae* species in birds (Weiner et al. 1979a). Fecal contamination caused by waterfowl (*Anas platyrhynchos*) polluted water to such an extent that a swimming beach in Wisconsin had to be closed (Stanbridge et al. 1979).

The effects of fish farms on chemical water quality variables tended to have a pattern of two peaks during the growing season, one in July and another in August (Section 3.13). The two peaks were to some extent observable with the increase in total coliform bacteria but not so markedly with the increase of presumptive fecal streptococci. The main load of these bacteria and of phosphorus and nitrogen was released to the watercourse during July, August and September. The minima observed between the peaks may be due to stress in the fish population as a result of maximum water temperature and reduction in oxygen saturation.

Presumptive fecal streptococci and total coliform bacteria appeared to multiply at the fish farms, because their concentrations were regularly much lower in the influent than in the effluent waters during the growing season. The diurnal variation of their concentrations (Section 3.12) cannot explain the observed difference. Though the contamination of fresh feed cannot explain the contamination of effluent water always and at both the farms, its hygiene requires further studies. The multiplication of presumptive fecal streptococci and total coliform bacteria occurred in the alimentary tract of fish and probably also in the sediment. This is in agreement with the findings of the occurrence of *Enterobacteriaceae* and *Aeromonas* in the intestinal content of freshwater fish (Trust and Sparrow 1974, Souter et al. 1976, Yoshimizu et al. 1976, Allen et al. 1983, Sugita et al. 1983) and with the findings of the occurrence of *Streptococci* in the alimentary tract of fish (Trust and Sparrow 1974, Kvasnikov et al. 1977).

Trust and Sparrow (1974) regarded salmonid fish in lakes as transient carriers of coliforms and enterococci. Kvasnikov et al. (1977) regarded lactic acid bacteria, such as *S. faecalis, S. faecium* and *S. bovis,* as part of the normal flora of cyprinoid fish. Yoshimizu et al. (1976) did not find *Streptococcus* species in salmonid fish reared in freshwater, but Sugita et al. (1983) occasionally found high quantities of these bacteria in different fish species living in a river. High concentrations of *Enterobacteriaceae* and of the *Vibrio-Aeromonas* group were assayed in fish living in a river (Sugita et al. 1983). The differences in isolation and identification methods can explain to some extent the

discrepancies between these investigations, but more likely the fish species and particularly the environmental conditions affect the multiplication of bacteria in the alimentary tract of fish (Geldreich and Clarke 1966, Lesel 1981).

The proliferation of thermotolerant coliform bacteria at fish farms was not common. Elevated concentrations tended to occur simultaneously in the influent and in the effluent. The occasional negative values of the increase coefficients indicate that the effluent sample was taken while more contaminated water was entering the farm. The elevated concentrations in June 1981 in the influents can be explained by contamination due to runoff waters, as this month was exceptionally rainy (120 mm at the Rastu meteorological station, 10 km from the Tyyrinvirta farm and 190 mm at the Vesanto meteorological station, 15 km from the Äyskoski farm). The maximum concentration of thermotolerant coliforms in the effluent at the Äyskoski farm was observed on the 30th of June. This day and the preceding day were rainy, with 18 to 19 mm of precipitation at the Vesanto station. The effects of accumulation of cattle feces on pastures (Thelin and Gifford 1983), increased use of summer cottages and rainfall may together explain the increased contamination of water in July and August at Tyyrinvirta.

Ruane et al. (1977) observed remarkable increases of thermotolerant coliform bacteria at a catfish farm. The high water temperature, up to 29 °C in their study, probably explains the marked increase in the catfish farm although no such remarkable increase was observed in the present study.

The confirmation tests on bacteria which were isolated during the diurnal variation experiment (Section 3.12) implied that thermotolerant coliforms were mainly presumptive *E. coli* bacteria, whereas presumptive fecal streptococci were not confirmed. The discrepancy between the behaviour of presumptive fecal streptococci, total coliform bacteria and thermotolerant coliform bacteria in the correlation analysis (Section 3.11), the diurnal variation study (Section 3.12) and the seasonal variation study (Section 3.13) all indicate that presumptive fecal streptococci and total coliform bacteria are not reliable indicators of fecal contamination by homoiothermic animals.

In order to test the hypothesis that neither presumptive fecal streptococci nor total coliform bacteria indicate fecal contamination by homoiothermic animals at fish farms, but that thermotolerant coliform bacteria are reliable indicators, it was necessary to characterize and identify bacteria from different fecal indicator groups (Sections 3.2 to 3.4).

3.2 Characteristics of presumptive fecal streptococci

3.21 Results

Presumptive fecal streptococci were isolated during the growing season from the waters at the Tyyrinvirta farm. The intention was to isolate 412 bacterial strains forming typical colonies on KF Streptococcus agar, but unsuccesfull isolations, especially from the effluent water, were common (Fig. 3, Table 12). Only six of the isolates were catalase positive and were discarded from further testing. The confirmation test for esculin hydrolysis on bile- and azide containing medium at an incubation temperature of 44 °C showed clear differences between strains isolated from the influent and the effluent.

The confirmed strains were identified with the API 20 Strep system (Table 13). About 60 % of the identifications proved to be enterococci. Of these, 34 strains were isolated from the influent and 3 strains from the effluent of Tyyrinvirta farm. Viridans streptococci were not found. The identification of *S. lactis* is doubtful because this organism should not grow at 45 °C or tolerate salt.

A few bacteria negative in the confirmation test but otherwise showing reactions typical of enterococci were isolated both from the influent and the effluent (Table 14, biotype 1). The strains negative in the confirmation test were facultatively anaerobic (except two obligately aerobic strains) oxidase negative (except one strain) and nonmotile (except one strain). The vast majority of strains were Gram positive or variable and cells were either oval or short rods and often formed short chains. However, the interpretation of microscopic examinations was difficult. The most frequently isolated biotype, especially from the effluent samples, was number 5. Number 2 was also common. Many of the effluent strains could produce acid from sorbitol, but only one of the influent strains utilized sorbitol.

Table 12. Isolation and confirmation of presumptive fecal streptococci from influent and effluent waters at fish farms during the growing season in 1982 (six sampling dates).

Sample	Total		Unsuccesfull isolations		Catalase positive,	Esculin positive	
	no.	%	no.	%	no.	no.	%
Influent	161	39	11	6.8	4	54	34
Effluent	251	61	51	20	2	9	3.6
Total	412	100	62	15	6	63	15

Table 13. Identity and biochemical characteristics of confirmed fecal streptococci from fish farm waters isolated from KF Streptococcus agar.

API 20 Strep identification	No.	Growth			Acid from		
		45 °C	6.5 % NaCl	10 °C	sorbitol	arabinose	lactose
S. faecalis	17	+[1]	16/17[2]	+	13/17	—	16/17
S. faecium	9	+	+	+	2/9	8/9	+
S. faecium durans	8	+	+	+	1/8	—	+
S. faecium durans/ S. faecalis	1	+	+	+	+	—	+
S. faecium/S. lactis	1	+	+	+	—	—	+
S. faecium durans/S. lactis	1	Not tested					
S. lactis	4	1/4	2/4	+	—	—	+
S. lactis	1	Not tested					
Aerococcus sp.	1	+	+	+	+	—	+
Unidentified	8	3/8	5/8	7/8	—	1/8	6/8
Lost	10						
Total	61						
Positive	49	84 %	88 %	98 %	37 %	18 %	94 %

[1] + or — = all tested strains positive or negative, respectively
[2] positive/tested

Table 14. Characteristics of presumptive fecal streptococci from fish farm waters which were negative in the esculin confirmation test (catalase negative strains).

Source	Biotype	Strains		Growth			Acid from		
	no.	no.	%	45 °C	6.5 % NaCl	10 °C	sorbitol	arabinose	lactose
Influent	1	6	6.7	+[1]	+	+	—	2/6[2]	+
	2	19	21	+	—	+	—	3/19	14/19
	3	5	5.6	—	+	+	—	2/5	2/5
	4	2	2.2	+	—	—	—	—	1/2
	5	21	23	—	—	+	1/21	5/21	14/21
	6	7	7.8	—	—	—	—	—	3/7
	Lost	30	33						
	Total	90	100						
	Positive	60		45 %	18 %	85 %	1.7 %	20 %	67 %
Effluent	1	7	3.6	+	+	+	1.7	5/7	+
	2	28	15	+	—	+	3/28	9/28	17/28
	3	7	3.6	—	+	+	1/7	3/7	6/7
	4	2	1.0	+	—	—	—	1/2	1/2
	5	71	37	—	—	+	12/71	19/71	49/71
	6	4	2.1	—	—	—	—	3/4	+
	Lost	74	38						
	Total	193	100						
	Positive	119		31 %	12 %	95 %	14 %	34 %	71 %
Total		283	100						
	Positive	179		36 %	14 %	92 %	10 %	29 %	69 %

[1] + or — = all tested strains positive or negative, respectively
[2] positive/tested

3.22 Evaluation

KF Streptococcus agar was originally reported to support the growth, as typical colonies, of all fecal streptococci, whereas other species and genera of the *Streptococcaceae* family grew not at all or formed atypical colonies (Kenner et al. 1961). Later on, however, other bacteria have been reported to interfere: lactobacilli, aerococci and pediococci (Raibaud et al. 1961, Mundt 1976), *Staphylococcus aureus* (Mossel 1964), pseudomonas, filamentous Gram negative rods and micrococci (Slanetz and Bartley 1964) and pin point colonies of which sometimes only a few belong to group D (Havelaar and Engel 1981).

The confirmation with esculin hydrolysis on a medium containing azide and bile at an elevated incubation temperature (Engel and Soedirman

1976, Havelaar et al. 1982) has not had a long tradition. However, growth at 45 °C, tolerance of azide and bile and esculin hydrolysis have for a long time been used for the specific enumeration of enterococci (Hartman et al. 1966). The requirement of all these characteristics in one test may decrease the yield of enterococci and, particularly, of the other fecal streptococci (Hartman et al. 1966). Engel and Soedirman (1976) observed that the growth of *S. bovis* and *S. equinus* was restricted even on a non-selective medium at 45 °C. *S. mitis* and *S. salivarius* are negative in this confirmation test (Deibel and Seeley 1974).

In practice, the use of KF Streptococcus agar for the primary cultivation and BEA agar at 44 °C for the confirmation leads to the predominance of enterococci. The exclusion of buccal streptococci, *S. mitis* and *S. salivarius,* is acceptable due to their questionable value as fecal indicators (Clausen et al. 1977, Beaudoin and Litsky 1981). For special studies on recent fecal pollution from farm animals by enumeration of species that die rather rapidly in water, such as *S. bovis* and *S. equinus* (Geldreich and Kenner 1969), other methods must be used (Oraqui and Mara 1984).

In the present study measurements on KF Streptococcus agar revealed remarkable increases of bacteria at fish farms. Pin-point colonies were common. Catalase positive strains were rare, but the vast majority of strains, especially from the effluent water, failed in the confirmation with BEA agar at 44 °C. Some of the confirmed strains remained unidentified in the API 20 Strep identification (Table 13). Only a minor fraction of the negative confirmations could have been false negative enterococci (Table 16, biotype 1, Deibel and Seeley 1974). Biotype 4, to which *S. salivarius, S. mitis, S. bovis* and *S. equinus* belong, was also rare. It therefore seems that when studying fish farm waters it is the primary cultivation medium which fails, rather than the confirmation test.

Because members of the genus *Lactobacillus* are only partially inhibited on KF Streptococcus agar (Raibaud et al. 1961), because both coccal and rod-shaped lactic acid bacteria are observed in high concentrations in the intestinal contents of fish (Kvasnikov et al. 1977) and because the interpretation of the Gram stain was not reliable in this study, the occurrence of the genus *Lactobacillus* among the non-confirmed strains cannot be excluded. Indeed, the descriptions of lactobacilli (Rogosa 1974) would allow many species to be placed into all the biotypes except number 1 (Table 14) if the salt tolerance test, of which the result is not known for all *Lactobacillus* species, was disregarded.

Five strains negative in the confirmation test but allocated to biotype 1 (Table 14), were also lacking in acid production from sorbitol and arabinose thus preventing their identification as *S. faecalis* or *S. faecium* (Deibel and Seeley 1974). The majority of biotype 2 strains differed from *S. uberis* in the sugar fermentations. Biotype 3 strains conformed with the *Streptococcaceae* genera *Leuconostoc, Pediococcus* and *Aerococcus* and with one species of the genus *Lactobacillus.* Biotype 4 conformed with *S. bovis, S. equinus, S. mitis, S. salivarius* and some *Lactobacillus* species. Biotype 5 was in accord with *S. lactis, S. gremoris, Leuconostoc, Pediococcus* and some *Lactobacillus* species. Some of the *Lactobacillus* species could have given the reactions of biotype 6, exept for 3 strains which were different in their sugar fermentations.

In a study by Brodsky and Schiemann (1976), all the strains tested were Gram positive cocci. The cell morphology agreed with streptococci in a later examination as well (Sibakov and Niemelä 1979). In these two studies strains isolated from different media, including KF Streptococcus agar, proved to have biochemical properties intermediate between the descriptions of species by Deibel and Seeley (1974). Indeed, Jones (1978) and Beaudoin and Litsky (1981) remarked that there remains a significant number of streptococci that fail to conform to the reaction pattern of any recognized species.

In the present study the confirmation test did not result in so many potentially false negative enterococci, *S. bovis* or *S. equinus*, that it could have affected the interpretation of the hygienic state of the fish farm waters. The confirmation test proved to be useful in showing that the influent waters were slightly contaminated with feces of homoiothermic animals or humans and that the increase of presumptive fecal streptococci at fish farms was due to the increase of bacteria not related to fecal contamination by humans or homoiothermic animals.

3.3 Identity of coliform bacteria

3.31 Results

Total coliform bacteria were isolated from samples of influent, effluent, sediment and fish feces. Identification with the API 20 E system revealed differences in species composition between these sources (Table 15).

When the sources were pooled, the species compositions at the two farms were significantly different (Table 16). *E. coli* was more common and

Table 15. The species composition of total coliform bacteria in different environments at fish farms.

Genus/Species	Division into species of isolates from different sources (%)							
	Influent		Effluent		Sediment		Fish feces	
	1[1]	2[2]	1	2	1	2	1	2
E. coli	34	20	20[3]	3.2	24[4]	4.0	9.7	5.4
Citrobacter	8.3	15	15	26	21	31	11	14
Other Enterobacteriaceae	28	37	16	20	14	19	13	41
Unidentified oxidase —ve	10	20	14	27	17	19	37	30
A. hydrophila	13	2.2	21	14	19	19	3.2	2.7
Others	7.5	6.5	14	9.2	5.5	7.5	26	8.1
Total	100	100	100	100	100	100	100	100
Total no. of strains	120	46	237	250	274	227	62	37

[1] The Tyyrinvirta farm
[2] The Äyskoski farm
[3] 24 of the 48 strains *E. coli* 4
[4] 58 of the 66 strains *E. coli* 4

Table 16. χ^2 tests of the species composition of coliform bacteria (Brant-Snedecor's formula). d.f. = degrees of freedom.

Comparison	d.f.	χ^2
Tyyrinvirta — Äyskoski	5	101^{***}
Influent — Effluent	5	52.4^{***}
Feces — Sediment	5	51.1^{***}
Feces — Effluent	5	27.5^{***}
Sediment — Effluent	5	13.0^{*}
Early June — Later June	5	26.9^{***}

Citrobacter less common at the Tyyrinvirta farm than at the Äyskoski farm. When the bacteria isolated from the two farms were pooled, the species compositions in the influent and the effluent waters were different (Table 15 and 16). In the influent the percentages of *E. coli* and other *Enterobacteriaceae* were higher and the percentages of *Citrobacter* and *A. hydrophila* were lower than in the effluent. The species composition was different between fish feces and sediment, as well as between fish feces and effluent. The differences between sediment and effluent species were less significant. The percentages of *Citrobacter* and *A. hydrophila* were higher in the sediment and effluent samples than in the fish feces, which contained many unidentified organisms.

Table 17. The species composition of total coliform bacteria at fish farms during the summer.

	Division into species of isolates from different dates (%)							Total no. of strains
	E. coli	Citrobacter	Other Enterobacter-iaceae	Unidentified oxidase —ve	A. hydrophila	Others	Total	
Tyyrinvirta farm								
June 15th	7.1	3.6	37	26	18	8.9	100	112
29th	12	12	21	26	7.1	22	100	113
July 14th	35	15	15	10	23	2.6	100	115
28th	26	24	8.5	12	16	12	100	140
August 10th[1]	21	23	15	10	25	7.2	100	110
28th	38	16	9.8	15	14	8.8	100	103
Äyskoski farm								
June 9th	1.2	8.5	27	3.7	37	22	100	82
22nd[1]	8.1	8.9	15	27	27	14	100	124
July 7th	2.2	5.6	22	54	12	3.3	100	90
20th	1.0	38	24	33	2.9	1.0	100	105
August 4th	3.6	52	20	19	0	4.8	100	84
18th	13	46	24	12	3.2	3.2	100	95

[1] No sample from fish feces

The species composition changed during the course of the summer (Table 16 and 17). The incidence of *E. coli* was higher in July and August than in June at the Tyyrinvirta farm. *E. coli* 4 was common among total coliforms at the Tyyrinvirta farm in sediment and effluent samples taken in July and August. It occurred in one sample of fish feces at the Tyyrinvirta farm. Only two *E. coli* 4 strains were isolated from the Äyskoski farm as total coliforms. At both of the farms the incidence of *Citrobacter* among isolates was high, while the total number of coliform bacteria increased markedly (Fig. 9). At the Äyskoski farm there was a decrease in the percentage of *A. hydrophila* during the autumn. A similar decrease was not observed at the Tyyrinvirta farm.

The identification of thermotolerant coliform bacteria revealed that *E. coli* was the dominant strain in all environments (Table 18).

At the Tyyrinvirta farm only pelleted dry feed was used, whereas at Äyskoski a minor part of the feed was fresh. The majority of the non-thermotolerant coliform bacteria from pelleted feed were primarily identified as *Enterobacter* species (Table 19), but many of them did not differ clearly from *Serratia* or *Klebsiella*. *Citrobacter* species were often isolated as non-thermotolerant coliform bacteria. Of the thermotolerant coliform bacteria, the vast majority were *E. coli* strains.

Coliform bacteria isolated from the samples of fresh feed were identified. Of the 20 total coliform bacteria, one was *E. coli* 1, three were *C. freundii* 1 and 16 were unidentified oxidase negative strains. Of the ten bacteria isolated as thermotolerant coliform bacteria, eight were *E. coli* 1, one was *E. coli* 3 and one remained unidentified.

Of the identifications of total coliform bacteria, 32 % were sufficiently reliable to give an identification percentage of at least 99 (Table 20). In the case of thermotolerant coliform bacteria equal reliability of identification was achieved for 67 % of the strains (Table 21). The 1244 strains of total coliform bacteria produced on average 3.6 identical patterns of biochemical reactions, but there were differences between taxa in the homogenity (Table 20). *A. hydrophila* and *H. alvei* were usually reliably identified.

Table 18. The occurrence of *E. coli* among thermotolerant coliform bacteria at fish farms (12 sampling dates).

Sample	Strains identified as E. coli			
	Tyyrinvirta farm		Äyskoski farm	
	no.	%	no.	%
Influent	38	79	11	100
Effluent	84	86	48	92
Sediment	31	97	9	100
Fish feces	19	95	7	100
Fresh feed[1]			9	90

[1] from 1 sample of ground small fish

Table 19. The species composition of coliform bacteria in 15 samples of pelleted fish feed.

Genus/Species	Non-thermotolerant		Thermotolerant	
	no.	%	no.	%
Enterobacter[1]	101	58	3	8.6
Citrobacter	22	13	0	
Serratia	13	7.5	0	
Acinetobacter	5	2.9	0	
Klebsiella	4	2.3	3	8.6
E. coli	1	0.6	27	77
Unidentified				
oxidase −ve	15	8.1	2	5.7
oxidase +ve	13	8.1	0	
Total	174	100	35	100

[1] Many of the strains were intermediate with other *Enterobacteriaceae* genera

3.32 Evaluation

The identification results showed that the analysis of thermotolerant coliform bacteria measured mainly the occurrence of *E. coli* at fish farms. In the less selective method of total coliform bacteria, *E. coli* was often overgrown by other *Enterobacteriaceae* species, *A. hydrophila* and many unidentified species which, however, produced typical coliform colonies on LES Endo agar.

Differences are observed in the species composition of bacteria isolated and identified with similar methods from different aquatic sources (Table 22). The percentages of *E. coli* and *Klebsiella* are higher and the percentage of *Citrobacter* lower in domestic sewage than at fish farms. The percentages of *Klebsiella* and *Enterobacter* are higher in the waste waters from the textile and food industries than at fish farms, but the percentage of *Citrobacter* in these wastes is closer to their percentage at fish farms than in domestic sewage. The effluents of forest industry are rich in *Klebsiella* and *Enterobacter*, which are much less frequently isolated from fish farms.

It is interesting to note that although many similarities were recorded between the bacterial flora in this study and the flora associated with a freshwater rainbow trout farm in England, *Citrobacter* was not found in the latter (Allen et al. 1983). Either the flora is truly different, or the

Table 20. Identity of strains isolated from LES Endo agar according to the API 20 E (1983). Of the strains isolated, 29 were lost before identification.

Genus/Species	Total		No. of different		No. of strains giving identification %				Low discrimination	Unidentified
	no.	%	API codes	(1)	≥ 99.9	≥ 99	≥ 95	≥ 80		
C. freundii 2	168	21	20	(8.4)	1	47	119	1		
C. freundii 1	91		15	(6.1)			1	54	36	
Other Citrobacter	2		1	(2.0)			2			
A. hydrophila	199	16	41	(4.9)	186	3	10			
E. coli 4	89	15	5	(18)			1	64	24	
E. coli 1	85		12	(7.1)	39	13	32	1		
E. coli 2	10		5	(2.0)			8		2	
E. coli 3	6		3	(2.0)		1	4	1		
K. pneumoniae	65	20	6	(11)		21	27	17		
K. oxytoca	14		3	(4.7)			13		1	
K. ozaenae	3		3	(1.0)					3	
E. cloacae	44		7	(6.3)		32	9	3		
E. agglomerans 4	19		5	(3.8)			3		16	
Other E. agglomerans	3		2	(1.5)			2		1	
E. aerogenes	7		4	(1.8)			4	2	1	
E. sakazakii	2		1	(2.0)			2			
H. alvei	50		13	(3.9)	21	23	6			
S. fonticola	30		8	(3.8)		8	12	3	7	
Other Serratia	7		5	(1.4)			4		3	
Other identified	6	0.5	5	(1.2)					6	
Unidentified										
oxidase −ve	261	21	114	(2.3)						261
oxidase +ve	83	6.7	66	(1.3)						83
Total	1244	100	344	(3.6)	20 %	12 %	21 %	12 %	8.0 %	28 %

1) Number of strains per API code on average

Table 21. Identity of strains isolated from mFC agar according to the API 20 E (1983). Of the strains isolated, 3 were lost before identification.

Genus/Species	Total		No. of different		No. of strains giving identification %				Low discrimination	Unidentified
	no.	%	API codes	(1)	≥ 99.9	≥ 99	≥ 95	≥ 80		
E. coli 1	223	89	25	(8.9)	163	20	37	3		
E. coli 3	12		5	(2.4)		2	10			
E. coli 2	11		3	(3.7)			8		3	
E. coli 4	10		3	(3.3)	4		6			
K. pneumoniae	5	2.4	1	(5.0)		5				
S. fonticola	1		1	(1.0)				1		
E. agglomerans 2	1		1	(1.0)			1			
Unidentified										
oxidase −ve	24	8.4	16	(1.5)						24
Total	287	100	55	(5.2)	58 %	9.4 %	22 %	1.4 %	1.0 %	8.4 %

1) No. of strains per API code on average

Table 22. The species composition of coliform bacteria from different Finnish aquatic environments. Bacteria were isolated as typical coliform colonies from LES Endo agar and identified with the API 20 E system and the API Analytical Profile Index (the 1983 edition for fish farm strains and older editions for the others), except the strains from forest industry, which were identified with the Enterotube system.

Genus/Species	Fish farms		Domestic sewage[1]		Food & textile industry[2]		Forest industry[3]	
	no.	%	no.	%	no.	%	no.	%
Citrobacter	261	21	5	3.0	48	15	0	0
A. hydrophila	199	16						
E. coli	190	15	61	37	18	5	36	10
Klebsiella	82	6.6	24	15	139	42	185	52
Enterobacter	75	6.0	9	5.5	67	20	65	18
H. alvei	50	4.0					1	0.3
Serratia	37	3.0					1	0.3
Other identified	6	0.5	16	10	5	1.5		
Unidentified			49	30	54	16		
oxidase −ve	261	21					71	20
oxidase +ve	83	6.7						
Total	1 244	100	164		331	100	359	100

[1] Anonymous 1981
[2] Anonymous 1982
[3] Niemelä and Mentu, personal communication

identification as *C. freundii* 1 or 2 in this work was erroneous. Incorrect identification seems possible because 72 % of the strains identified as *C. freundii* 2 were citrate negative while none of the strains identified as *C. freundii* 1 produced hydrogen sulphide and 64 % were citrate negative. Souter et al. (1976) identified some of the bacteria isolated from fish as *C. freundii* when using the methods of Edwards and Ewing (1972) or the API 20 E system. *Citrobacter* was a common isolate from frogs when bacteria were identified with the API 20 E system (Hird et al. 1983). Allen et al. (1983) measured 124 unit characters and analysed the results by numerical taxonomy, which may give different identification results from the API 20 E system.

The taxonomy of *Citrobacter* used in the API 20 E system differs from the conventional division into species (Table 23). There are differences in the interpretation of the results from the citrate, hydrogen sulphide, urea and indole reactions. The less sensitive test for the decomposition of urea in the API 20 E explains the difference in this test. In the present study, the citrate negative strains identified as *C. freundii* 1 and 2 were more common than in the API data base. The reactions of *C. freundii* 2 in the inositol and sorbitol tests were reversed compared to those in the API data base, and were in discrepancy with the results of the other authors (Table 23).

The API 20 E identification is based on the use of a computer (Gyllenberg 1965, Lapage et al. 1973). In 89 to 97 % of cases, API 20 E identification of enterobacteria yielded the same identification of laboratory strains as conventional methods in a trial including 40 microbiologists testing ten different commercial identification kits (Fung et al. 1983). Genus-species identification of clinical isolates was 97.3 % reproducible, but only 55.5 % of the strains gave identical reactions in all 20 of the API 20 E biochemical tests on repeat testing by two technicians at different times (Butler et al. 1975). Austin et al. (1981) obtained the same identification of coliform bacteria from aquatic environment with API 20 E rapid identification kits as with conventional methods in 83 % of cases. They remarked that although commercially available identification kits are acceptable for identifying commonly occurring, well-defined taxa of the family *Enterobacteriaceae* found in clinical specimens, they do not yield reliable results when used to identify atypical strains of incompletely defined taxa commonly isolated from the aquatic environment, e.g. *C. freundii.* Schindler et al. (1979) concluded that the differences in the computer and conventional identifications of *Citrobacter*-like organisms is caused by confused taxonomy.

Dr. F. Gavini (INSERM, France) identified and Dr. S. Niemelä (University of Helsinki, Department of Microbiology) measured the maximum growth temperatures of some of the most common isolates identified as *C. freundii* by the API 20 E system in this study. The three *C. freundii* 1 strains were identified as *Enterobacter amnigenus* (Izard et al. 1981) and had maximum growth temperatures below 40.5 °C. The two *C. freundii* 2 strains were accepted as *C. freundii* and had maximum growth temperatures exceeding 43 °C.

Table 23. Characteristics of *Citrobacter* species.

Tests	Frequency of each characteristic in different strains (%)									Occurence of each characteristics		
	This study			API 20 E (1983)			Edwards & Ewing (1972)			Brenner (1984)		
	C. freundii 1	C. freundii 2	Others[1]	C. freundii 1	C. freundii 2	Others[1]	C. freundii	IND+CIT+	IND−CIT−	C. freundii	C. diversus	C. amalonaticus
ONPG	100	99	100	96	86	97				+	+	+
ADH	30	20	0	18	36	50	44	64	10	d[2]	d	d
LDC	0	0	0	0	0	0	0	0	0	−	−	−
ODC	90	39	100	32	36	98	17	90	6	d	+	+
CIT	35	28	100	58	72	95	90	93(+7)[3]	0	+	+	d
H_2S	0	100	0	1	99	0	82	21	55	d	−	−
URE	0	0	0	1	5	0	69	82	39	d	d	d
TDA	0	0	0	0	0	0						
IND	7	1	100	6	8	98	7	100	0	−	+	+
VP	0	0	0	0	0	0	0	0	0	−	−	−
GEL	0	0	0	0	0	0	0	0	0	−	−	−
GLU	100	100	100	100	100	100	100	100	100	+	+	+
MAN	100	100	100	99	99	99	100	100	100	+	+	+
INO	0	99	0	10	8	2	3	0	3	−	−	−
SOR	98	2	100	95	89	92	98	95	94	+	+	+
RHA	100	99	100	84	90	94	99	100	100			
SAC	22	7	0	68	40	24	15	10	35	d	d	d
MEL	92	90	0	66	73	0						
AMY	98	96	0	38	64	96						
ARA	100	100	100	96	95	95	100	90	100	+	+	+
OX	0	0	0	0	0	0	0	0	0	−	−	−
No. of strains	91	168	2				582	39	31			

[1] C. diversus/amalonaticus
[2] d = variation between strains
[3] brackets indicate delayed reaction

When those API 20 E profiles giving identification as *C. freundii* 1 according to the API 20 E (1983) are compared to the species description of *E. amnigenus* (Izard et al. 1981), 14 % of the strains give exactly the same reactions as *E. amnigenus* and if arginine dihydrolase negative and citrate negative strains are accepted 84 % of the *C. freundii* 1 strains can be allocated to *E. amnigenus.* If the citrate utilization reaction is rejected as unreliable, lack of hydrogen sulphide production and a positive reaction in the ornithine decarboxylase test still separate these strains from typical *C. freundii* (Brenner 1984). All the strains identified as *Enterobacter cloacae* in this study were positive in the Voges-Proskauer test, whereas all the strains resembling *E. amnigenus* were negative. According to Izard et al. (1981) 98 % of *E. amnigenus* strains are positive in this test.

The identification to the genus *Serratia* was not very reliable. All the strains identified as *S. fonticola* were in accordance with the species description of Gavini et al. (1979), except that two thirds of the strains were lysine decarboxylase negative. All the strains form LES Endo agar identified as *S. fonticola* were mannitol-, inositol- and β-galactosidase positive, but urea-, tryptophane deaminase-, indole-, hydrogen sulphide-, arginine dihydrolase- and oxidase negative. The strain identified as *S. odorifera* differed from *S. fonticola* strains in producing indole. One thermotolerant strain was identified as *S. fonticola,* which is in discrepancy with the findings of Leclerc et al. (1983) on the maximum growth temperature of this species.

The *E. coli* biotype 4, producing hydrogen sulphide, was the most frequently isolated *E. coli* among total coliforms. This distinguishing characteristic is determined by a plasmid and has been observed in many laboratories from the beginning of nineteen-seventies (Ørskov 1981). The identification to the biotype *E. coli* 4 was not reliable (Table 20). The fecal origin of these strains can be questioned in this study, since they were isolated mainly from the sediment and effluent samples from the Tyyrinvirta farm and only once (as thermotolerant coliforms) from the influent water, where *E. coli* 1 was the dominant *E. coli* biotype. *E. coli* 4 was seldom isolated as a thermotolerant coliform.

Enterobacter agglomerans was not common among the isolates, but its identification was problematical. The discrimination of *E. agglomerans* from the genera *Citrobacter, Klebsiella* and *Serratia* was often low.

Farmer III (1981) stated that, in the future, new species will probably be added to the *Citrobacter* and *Enterobacter* genera, making their separation increasingly difficult if they represent a continuum of evolution. He noticed that *Citrobacter* species are well adapted for growth and survival in the environment and that environmental strains do not resemble any of the currently defined species of *Enterobacteriaceae.* Environmental strains labeled *Citrobacter* sp. or *Enterobacter* sp. are, according to Farmer III, a heterogeneous group that will probably be divided into many new species.

In addition to low reliability of identification of many of the bacterial strains associated with fish farming, a considerable fraction remained unidentified. Therefore, the use of numerical taxonomic methods was considered necessary (Section 3.4).

3.4 Numerical taxonomy of coliform bacteria isolated from fish farms

3.41 Results

The identifications yieded 55 different biotypes from the 287 isolates from mFC medium and 344 different biotypes from the 1244 isolates from the LES Endo medium (Tables 20 and 21). The 13 most frequently occurring biotypes accounted for 47 % of the strains (three *E. coli* 1, two *E. coli* 4, two *A. hydrophila,* two *K. pneumoniae,* one *C. freundii* 1, one *C. freundii* 2, one *E. cloacae* and one unidentified biotype). (The bacterial names used refer to the API 20 E identification). The 143 biotypes containing at least two identical strains were taken into the preliminary cluster analysis.

The similarity matrix revealed three major clusters, *A. hydrophila, H. alvei* and a large cluster containing the majority of strains. The *A. hydrophila* cluster included two oxidase positive and one oxidase negative unidentified biotype. The *H. alvei* cluster included unidentified biotypes. More detailed examination was difficult with this set of data, although subclusters did occur (Fig. 12).

The limited number of tests hampered the use of Jaccard's coefficient of similarity, which excluded negative matches. Because only 21 tests were available, one test in difference means five % difference in similarity without negative matches, and with 11 negative matches one test in difference means ten % difference in similarity. In spite of these limitations, one subcluster of *E. coli* 1 strains and another of unidentified strains was formed (Fig. 12). The rest of the subclusters contained many common species. Neither *C. freundii* 1 nor *C. freundii* 2 formed a distinct cluster.

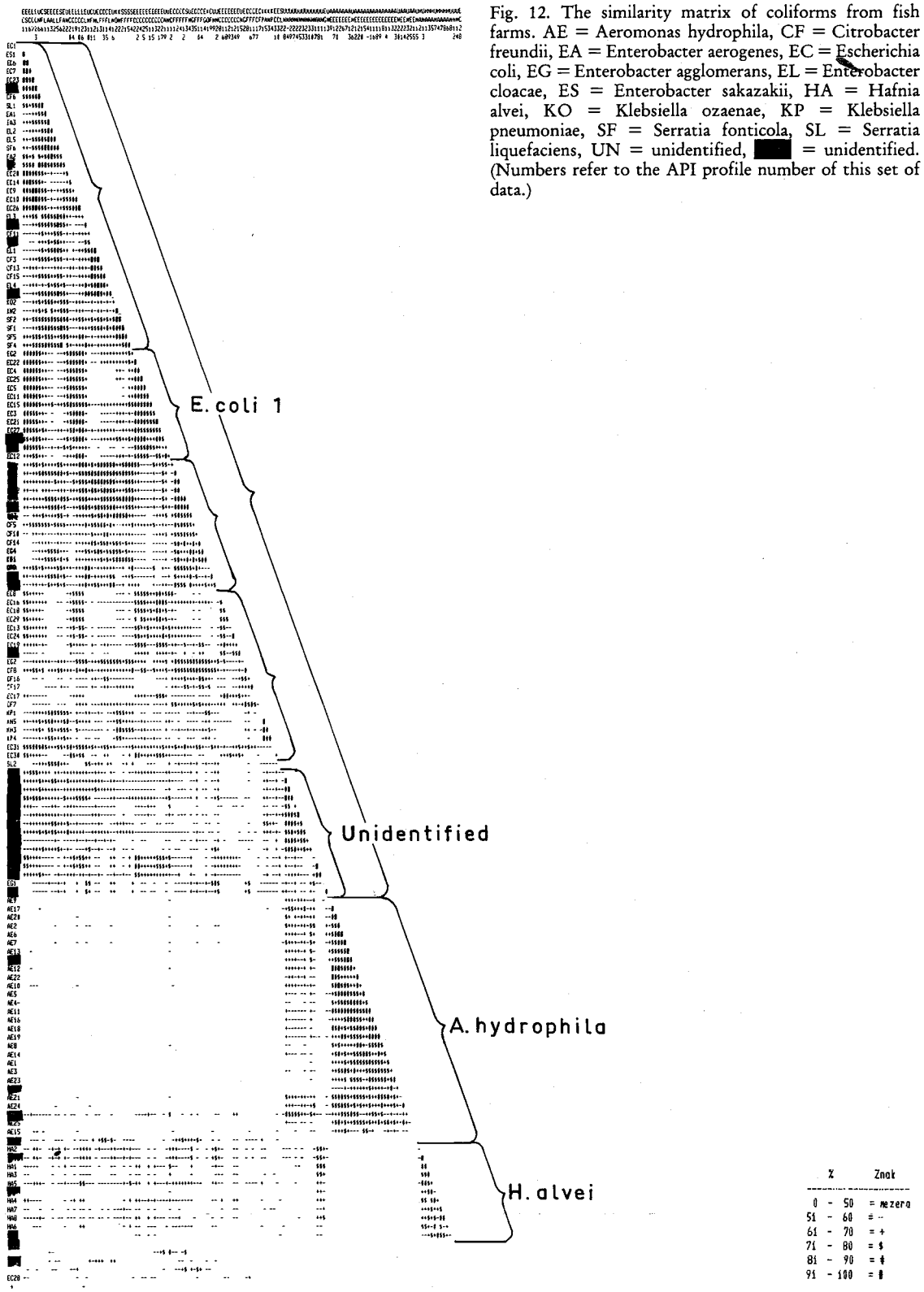

Fig. 12. The similarity matrix of coliforms from fish farms. AE = Aeromonas hydrophila, CF = Citrobacter freundii, EA = Enterobacter aerogenes, EC = Escherichia coli, EG = Enterobacter agglomerans, EL = Enterobacter cloacae, ES = Enterobacter sakazakii, HA = Hafnia alvei, KO = Klebsiella ozaenae, KP = Klebsiella pneumoniae, SF = Serratia fonticola, SL = Serratia liquefaciens, UN = unidentified, [redacted] = unidentified. (Numbers refer to the API profile number of this set of data.)

Table 24. Computer identification on the basis of data of Brenner (1984) and Izard et al. (1981), of strains yielding unreliable identification or remaining unidentified in the API 20 E (1983) system. Brackets indicate unacceptable identification percentage, below 80.

API identification	code	No. of strains	Computer identification[1]
Unidentified	1105553	71	Enterobacter 81 (E. intermedium 60)
	5344573	18	Kluyvera 98 (K. ascorbata 70)
	4115112	12	Hafnia alvei 99
	4114112	10	Hafnia alvei 98
	5044553	10[2]	Escherichia coli + E. coli inactive 98 (E. coli 80)
	1105173	6	(Enterobacter — Kluyvera 75)
	1305173	6	(Enterobacter 65) (E. amnigenus 34)
	5345151	5	Kluyvera 90 (K. cryocrescens 55)
	1105113	4	Hafnia alvei 98
	4115102	4	Hafnia alvei 95
	1305553	4	Enterobacter 96 (E. intermedium 79)
	2104513	4	Enterobacter — Escherichia — Citrobacter 83
	5315112	4	Enterobacter — Hafnia 100 (E. gergoviae 64)
	5145152	3	Hafnia — Escherichia — Kluyvera 93 (H. alvei 59)
	7347573	3	(Serratia 73) (S. odorifera 48)
Citrobacter freundii 1	1104553	32	(Klebsiella — Escherichia 61)
	1304553	13	(Serratia — Kluyvera — Enterobacter 75)
	3304553	12	Enterobacter amnigenus 93
	3104553	9	Enterobacter amnigenus 84
	3104573	6	Enterobacter 83 (E. amnigenus 72)
	1344573	6	Kluyvera 93 (K. cryocrescens 83)
	1004513	4	Escherichia — Enterobacter — Klebsiella 94
	1004573	4	Enterobacter — Klebsiella 85 (E. agglomerans 63)
Citrobacter freundii 2	1404553	73	Citrobacter — Klebsiella 86
	3704553	17	Arizona — Citrobacter — Enterobacter 100 (Arizona 69)
	1504553	16	(Citrobacter — Arizona 70) (C. freundii 40)
	3504553	15	Arizona — Citrobacter — Enterobacter 98 (Arizona 68)
	1504553	12	(Citrobacter — Arizona 70) (C. freundii 40)
	1704553	11	Citrobacter — Arizona 93 (C. freundii 53)
	1404513	8	Citrobacter — Escherichia 87 (C. freundii 77)
Escherichia coli 4	1444512	63	Escherichia coli inactive + E. coli 87 (E. coli inactive 74)
	1544512	23	(Escherichia — Yersinia 63)
	1444552	5	Escherichia coli inactive + E. coli 89
	5544572	4[3]	Escherichia — Kluyvera 95 (E. coli 69)
Enterobacter agglomerans 4	1004553	9	Klebsiella — Citrobacter 88 (K. pneumoniae ssp. ozaenae 71)
	1204553	6	Klebsiella — Citrobacter — Enterobacter 98 (K. pneumoniae ssp. ozaenae)
Serratia fonticola	1304753	7	Serratia — Klebsiella 90 (S. fonticola 72)
	1304773	6	(Enterobacter — Serratia — Kluyvera 75)
	5304753	6	Serratia fonticola 96
	1104753	5	Klebsiella — Serratia 96 (K. pneumoniae ssp. ozaenae 75)

[1] identification percentages or their sums are given
[2] 8 of the strains were thermotolerant
[3] thermotolerant strains

The taxonomy of the API 20 E (1983) was already out of date. The computer identification of commonly occurring strains that caused problems in the API 20 E identification was, therefore, perfomed using a different matrix (Table 24).

Many of the unidentified strains in the API 20 E system could be identified when the updated matrix was used (Table 24). However, strains intermediate between different genera occurred. *C. freundii* 1 was not confirmed as *Citrobacter. C. freundii* 2 seemed to be *C. freundii,* but the strains were atypical. The API identification as *E. coli* 4 was confirmed to be mainly the inactive *E. coli. E. agglomerans* 4 of the API identification seemed to be more probably *K. pneumoniae* ssp. *ozaenae,* although the discrimination was not good. The identification as *S. fonticola* remained uncertain.

3.42 Evaluation

The *Enterobacteriaceae* are ubiquitous in animals, plants, water and soil as saprophytes, symbionts, epiphytes and parasites (Brenner 1981). They have

been studied more thoroughly than any other group of organisms. The taxonomy of these bacteria has changed together with increasing knowledge and new methods of characterization. The general problem in bacterial taxonomy of the definition of species creates different opinions among taxonomists. How many biochemical differences are required to justify classification as a new species? How many tests should be done? Should all biochemical differences be given equal weight or should they be weighted differently? There are no simple answers to these questions, which is probably why a uniform concept for designating species has not been proposed (Brenner 1981).

Brenner regards DNA relatedness and numerical taxonomy as useful tools in bacterial taxonomy. Jones and Sackin (1980) stated that methods of numerical taxonomy have resulted in a greater insight into the classification and identification of the enterobacteria than any other modern method. They regard these methods as invaluable in ecological studies because they facilitate the handling of the large amount of data which such investigations generate. They also admit that identification of clusters of environmental isolates is difficult due to nontypical reference strains and the bias of medical microbiology influences.

In the present numerical classification, the strains identified as *A. hydrophila* with the API 20 E system formed a distinct cluster together with some unidentified strains. This is in agreement with the results of Allen et al. (1983), according to which *Aeromonas* species are clearly separated from enterobacteria associated with freshwater fish farming.

Strains identified with the API 20 E as *H. alvei* formed a separate cluster with some unidentified strains. In the study of Allen et al. (1983) a distinct *H. alvei* cluster was more closely related to *E. coli* and *Enterobacter aerogenes* than to the *Serratia* sp. cluster. Geipsson and Priest (1983) found that *H. alvei* strains were very similar to each other, but more related to *Yersinia* than other enterobacteria. Johnson et al. (1975) placed *H. alvei* between the two main clusters of *Enterobacteriaceae,* whereas Sakazakii et al. (1976) found it to be more related to *Citrobacter* and *Salmonella* than other enterobacteria. On the basis of DNA relatedness studies, Brenner (1981) predicted that *H. alvei* would be divided into two species and that some biotypes of *H. alvei* would be transferred to other genera of *Enterobacteriaceae,* possibly to the genus *Escherichia.* The source of species and the limited number of tests may explain the differences between different numerical taxonomy investigations and their inconcistency with DNA relatedness studies.

In spite of the limitations of the use of Jaccard's coefficient of similarity on a small number of tests, the main clusters and subclusters of *E. coli* and of unidentified strains demonstrate the utility of cluster analysis for the present data and encourage the further application of cluster analysis, e.g. the application of the simple matching coefficient of similarity in order to determine the positions of the unidentified strains in the clusters.

The comparison of the API 20 E (1983) identification and the computer identification using the matrix of the 9th edition of Bergey's manual (Brenner 1984) in the present study emphasizes the differences between the identifications because only the difficult strains were considered. The taxonomy of the latest edition of Bergey's manual was more suitable for the identification of many of these strains than the API 20 E (1983) identification. However, many of the strains remained intermediate between the *Enterobacteriaceae* genera. The API identification to *C. freundii* 1 was not reliable. Some of these bacteria were identified as *E. amnigenus* by Dr. F. Gavini (Section 3.32) and by the present computer identification.

4 DISCUSSION

4.1 Suitability of standard methods for the estimation of hygiene of waters affected by fish farms

Knowledge of the behaviour of indicator bacteria of fecal contamination has markedly increased during the past two decades, due mainly to increased identification of bacteria from different aquatic environments.

The use of fecal streptococci as an indicator of water hygiene has not hitherto been common elsewhere than in Finland. Recently, however, some authors have recommended fecal streptococci for this purpose. Miescier and Cabelli (1982) recommended enterococci as the indicator of municipal wastewater effluents because their numbers did not increase during the treatment and their reduction during treatment — including chlorination — was not as efficient as the reduction of *E. coli.* Fecal streptococci do not appear to multiply in Finnish wastewater treatment plants (Anonymous 1981). Clausen et al. (1977) and Miescier and Cabelli (1982) pointed out that,

because of their greater tolerance, fecal streptococci are safer indicators of viral contamination than thermotolerant coliform bacteria or *E. coli.* In his epidemiological studies on enteric viral infections associated with swimming, Cabelli (1981) observed enterococci to be the best indicator, followed by *E. coli,* whereas other indicators, e.g. total coliform and thermotolerant coliform bacteria, showed poor correlations with the frequency of gastrointestinal symptons.

Geldreich and Kenner (1969) stated that the true sanitary significance of fecal streptococci has been confused somewhat by controversies concerning procedures for quantification, definition of the group and differing concepts as to their occurrence in the aquatic environment and in fecal discharges. Beaudoin and Litsky (1981) regarded fecal streptococci as an important supplement to the coliform bacteria test for the assessment of the hygienic quality of water. They are useful because of their inability to multiply in waters, their good survival characteristics and the specificity of certain streptococcal species to particular vertebrate groups. Limitations to their use are caused by their occurrence in vegetation, the long incubation period in their enumeration and their lower density in human feces than the density of coliform bacteria.

An additional limitation to the use of fecal streptococci as indicators is their proliferation at fish farms. The simple, if time consuming confirmation test for enterococci, however, can be used to differentiate between contamination by human and homoiothermic animal feces and by fish farms. This two-step procedure clarifies the routine analyses in the confused state of taxonomy and ecology of streptococci from the aquatic environment. It was recently proposed that *S. faecalis* and *S. faecium* should be transferred to the genus *Enterococcus* (Schleifer and Kilpper-Bältz 1984). It has also been suggested that the closely related sorbitol- and arabinose negative strains, also observed in this study, be transferred into a separate species *S. durans* (Knight et al. 1984), ultimately *E. durans.* The confirmation test selects for the genus *Enterococcus.*

Total coliform bacteria are more suitable for the estimation of the quality of potable water than for the evaluation of surface water quality (Mack 1977). Much information on the ecology of many species of the *Enterobacteriaceae* family has been collected by determining the species composition of these bacteria in different aquatic environments. *E. coli* was present among the total coliform bacteria isolated from fish farms, but only as a minority. *A. hydrophila* increased markedly at fish farms. Only a minor fraction of *A. hydrophila* was observed as false positive coliforms, whereas the medium selective for *A. hydrophila* yielded much higher counts. Many species which occurred at fish farms in this study are known to be able to obtain transferable drug resistance if the fish are treated with chemotherapeutic agents at farms (Watanabe et al. 1971, Aoki 1975, Hayashi et al. 1982).

The determination of total coliform bacteria in water affected by fish farm effluents does not indicate fecal contamination by humans or homoiothermic animals. Because *E. coli* occurs only as a minor component among total coliform bacteria in these samples, the number of isolates must be large if it is required to determine the incidence of *E. coli.*

The determination of thermotolerant coliform bacteria measures mainly the occurrence of the genera *Escherichia* and *Klebsiella* (Dufour 1977, Niemelä et al. 1983). *Klebsiella* occurs in high numbers in industrial effluents rich in organic matter, which inconveniences the use of thermotolerant coliform bacteria as fecal indicators in some situations (Vlassoff 1977). In fish farm waters, however, *Klebsiella* were rare and the determination measured mainly the occurrence of *E. coli.* The occasional increase of *E. coli* at fish farms was regarded as a sign of fecal contamination of runoff to the farm waters from the banks contaminated by bird droppings. Another, less important source of contamination could have been contaminated fresh feed, because the one sample of fresh feed examined contained from 10^1 to 10^2 *E. coli* cells per gram.

Ruane et al. (1977) observed continuous increase of thermotolerant coliform bacteria at a catfish farm at an average water temperature of 28 °C. In their study the environmental conditions were so different from those of the present investigation that other organisms than *E. coli* could have been responsible for the increase.

In Finnish freshwater conditions, thermotolerant coliform bacteria are the only suitable fecal indicators for routine monitoring of waters affected by fish farms. However, the use of more than just one indicator generally gives more information. Due to the different environments for multiplication outside the intestines of humans and homoiothermic animals, and to the different survival characteristics of different organisms in aquatic environments (Clausen et al. 1977, Dufour 1977, Vlassoff 1977, Beaudoin and Litsky 1981), the combined use of thermotolerant coliform bacteria and fecal streptococci is advantageous when assessing the health risks caused by pathogenic organisms excreted in feces.

4.2 Suitability of the API 20 E identification system for bacteria isolated from aquatic environments

Although the API 20 E identification system was designed for medical laboratories it has been widely used for the identification of coliform bacteria from environmental samples. The majority of strains in the API data base originate from clinical laboratories. This may explain the remarkable fraction of environmental strains remaining unidentified, yielding unreliable identification or possibly even incorrect identification (Tables 20 and 22).

The severity of these problems depends on the clarity of the taxonomy behind the identification. Distinct species such as *H. alvei* (Greipsson and Priest 1983) are not problematical, whereas the genera *Enterobacter, Citrobacter* and *Serratia* contain many intermediate strains which are difficult to allocate to well defined species (Brenner 1981, Farmer III 1981). The addition of many new genera and species to the family *Enterobacteriaceae* in the last edition of Bergey's manual (Brenner 1984) promises more reliable identification of the environmental strains in future. In addition, the increased use of numerical taxonomy and genetic relatedness studies on environmental isolates will clarify the taxonomy (Brenner 1981). However, if related strains form a continuum without distinct clusters, like the *Citrobacter - Enterobacter* group (Farmer III 1981), the taxonomy remains complex. Intermediate strains are to be expected among *Enterobacteriaceae* cabable of genetic exchange (Jones and Sneath 1970).

The advantage of using a commercial identification system, such as API 20 E, is the standardized procedure. However, differences in the interpretation of reactions can occur. In this study the reliability of the citrate reaction was questioned because, according to the API 20 E (1983), *Citrobacter, Enterobacter* and *Klebsiella* were expected to give more positive reactions than were recorded. According to Brenner (1984) citrate utilization is even more common among these genera. By contrast, citrate positive strains of *H. alvei* accounted for 40 % of all the strains of this species, which was considerably more than expected (API 20 E 1983, Brenner 1984). Therefore, it seems probable that citrate positive strains among the *Citrobacter, Enterobacter* and *Klebsiella* isolates were really rare.

In this study an attempt was made to diminish the effect of the medical orientation of the data base of the API 20 E system by applying cluster analysis. The choice of tests and their number, however, were fixed to the API 20 E. Jones and Sackin (1980) pointed out the bias of medical microbiology in the taxonomy of environmental isolates, warned of non-relevant reference strains in culture collections, and recommended that at least 50 tests be carried out on the bacterial strains to be investigated using the methods of numerical taxonomy. In the data of this study, the number of tests, 21, was not adequate to separate closely related bacteria from each other. Inclusion of negative matches by using a simple matching coefficient instead of Jaccard's coefficient could improve the separation of strains in this set of data. The cluster formed by some unidentified strains demonstrates the usefulness of clustering methods for environmental samples.

The identification of *E. coli* 1 was reliable, whereas the identification of the equally frequently isolated *E. coli* 4 was not reliable in this study (Table 20 and 21). Brenner (1981) found that *E. coli* is a variable species with hundreds of different biotypes and that it is difficult to determine the biochemical boundaries of variable species. Mutations during storage and the existence of plasmids determining some of the characteristics used for taxonomic differentation, such as citrate utilization, urease production and hydrogen sulphide production in *E. coli,* further complicate the task of identification (Ørskov 1984).

Production of gas from lactose and production of indole, both at elevated incubation temperatures, are important criteria for the presumptive identification of *E. coli* (Bueschkens and Stiles 1984). Prolonged cold storage of *E. coli* strains was shown to lead to the loss of these characteristics. Indole production was more stable than the gas production characteristic. Brenner (1984) accepts indole- and even lactose negative strains as inactive *E. coli.* Therefore, simple and convenient methods used today cannot differentiate *E. coli* without a minor risk of error. Geldreich (1966) remarked that the IMViC-test should be used to identify a statistically significant sample of strains, not single strains, because of the variation of *E. coli* strains. This remark also applies to other tests.

Farmer III and Brenner (1977) discussed the importance of the concept of bacterial species in the analysis of water hygiene. They pointed out that many human pathogens have close relatives in aquatic environments. The separation of human pathogenic species from aquatic species is often possible only with extremely sensitive techniques such as DNA-DNA hybridization. Phenotypic differentiation of these species was not possible even with a great number of different tests. The authors expected that the separation would be

possible after determination of the DNA-DNA homology groups and selection of differential phenotypic tests on the basis of the determination. On the basis of DNA-DNA hybridization studies, Farmer III and Brenner (1977) concluded that *E. coli* is a phenotypically variable species and rather difficult to define, while *E. agglomerans* is not one species, but rather a cluster of a dozen species difficult to separate phenotypically. They warned that water quality standards based on named species will run into taxonomic difficulties and support the use of operational terms such as coliforms, fecal streptococci, enterococci and klebsiella-form.

The reasoning of Farmer III and Brenner (1977) supports the use of operational terms. However, the poor selectivity of the routine methods of water analysis, e.g. at fish farms, reveales the need to separate the species related to fecal contamination due to humans and homoiothermic animals from other bacteria by means of confirmation tests.

E. coli can be identified more reliably with the API 20 E identification system than with separate routine tests. Moreover, the API 20 E System provides insight into the species composition of coliforms as a whole. Usually, however, the simple tests give enough information for routine work. More reliable identifications of coliform bacteria are to be expected when the identification test kit designed for environmental strains becomes available (Gavini et al. 1983).

5 SUMMARY

This investigation was carried out with the aim of clarifying why fish farm effluents cause restrictions in water utilization. The data from 27 fish farms revealed that fecal indicator bacteria generally increased at freshwater farms.

In this study presumptive fecal streptococci were shown to increase markedly at fish farms. The increase showed diurnal variation, probably related to fish movement, and seasonal variation with high values from the middle of the summer to autumn. Bacteria of this group were present in the influent and in fish feed in low numbers, but in fish feces and sediment in much higher concentrations. However, the confirmation test was positive for only a minority of the strains isolated from the influent, and even fewer of the effluent strains were positive. The tolerance tests showed that false negative confirmation on the medium containing bile, azide and esculin at 44 °C could not explain the result. Therefore it was concluded that KF Streptococcus agar is not selective enough to be used for measuring fecal contamination by humans and homoiothermic animals at fish farms without laborous, costly and time-consuming confirmation. The same applies to m-Enterococcus agar, with which the increase of these bacteria at the Tyyrinvirta and Äyskoski farms was originally detected and which was used in the majority of analyses carried out at the other farms.

The increase in the total number of coliform bacteria at fish farms from June to autumn was evident. These bacteria were present in low numbers in pelleted fish feed (and in higher numbers in a sample of fresh feed), in varying quantities in influent water, and in higher amounts in fish feces and sediment. They multiplied in the alimentary tract of fish and probably also in the sediment.

The total coliform bacterial flora consisted of *Citrobacter*-like strains (of which many were *E. amnigenus* strains), *A. hydrophila, E. coli, Klebsiella, Enterobacter, H. alvei, Serratia* and a remarkable fraction of unidentified strains. *E. coli* was only a minor component of the total coliform bacteria of the influent, and its incidence in the effluent flora was still lower. This means that the determination of total coliform bacteria in order to assess fecal contamination caused by humans and homoiothermic animals in fish farm waters is impracticable due to the necessity of isolating a large number of strains for the confirmation tests. The number of *A. hydrophila,* an important member of the normal flora of fish and of waters, but also an opportunistic pathogen, increased markedly.

The determination of thermotolerant coliform bacteria measured mainly the occurence of *E. coli* in the fish farm environment, where *Klebsiella* were rare. The number of thermotolerant coliform bacteria was lower than the number of other fecal indicators at fish farms. The pelleted feed contained very few thermotolerant coliforms. The one studied sample of fresh feed was more hihgly contaminated. The level of contamination of the influent water varied, but the concentrations of thermotolerant coliforms usually increased simultaneously in the influent and in the effluent. However, occasionally these bacteria increased at the farm. This was interpreted as an indication of fecal contamination of runoff water at the farm by bird droppings. The low levels of thermotolerant coliform bacteria revealed that fecal contamination from fish farms occurs only seldom and at low

levels. They also indicated that the influent water at the Rautalampi watercourse was slighty contaminated by feces of humans or homoiothermic animals. The contamination was more pronounced at Tyyrinvirta than at Äyskoski.

Further research is needed to develop reliable laboratory methods for the storage and identification of fastidious bacteria observed as presumptive fecal streptococci. Only then will it be possible to arrive at a more satisfactory taxonomy for these bacteria. The application of numerical taxonomy to these bacteria could be a useful taxonomic tool. However, the methods of numerical taxonomy should be applied to a broad spectrum of lactic acid bacteria taken from different environments and tested carefully for morphological, physiological, biochemical and genetic characteristics (Jones 1978).

Re-evaluation of the bacteriological water quality criteria based on colony counts from the primary cultivation media is needed. Today, much information is available on the ecology of bacterial species counted in routine methods for the measurement of water hygiene in the aquatic environment, thus rendering re-evaluation possible.

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To Jorma and Aimo

Helsinki, July 1985

Maarit Niemi

TIIVISTELMÄ

Ihmisen ja tasalämpöisten eläinten ulosteiden aiheuttamaa saastutusta vedessä osoitetaan mittaamalla ulosteissa normaalisti esiintyvien bakteerien määrä. Rutiinitarkkailussa näiden bakteerien määrän todettiin kohonneen vesistössä, johon johdettiin vesiä kirjolohen kasvatuslaitoksilta. Tässä tutkimuksessa selvitettiin, lisääntyvätkö kalankasvatuslaitoksilla samat bakteerilajit, jotka ovat tyypillisiä ihmisen ja tasalämpöisten eläinten ulosteille.

Fekaalisten streptokokkien todettiin lisääntyvän huomattavasti veden lämmittyä ja lisääntymisen jatkuvan syksyyn. Varmistustestit kuitenkin osoittivat, että lisäyksen aiheuttivat muut lajit kuin ne, joita pidetään ulosteille ominaisina. Lisätestien avulla voitiin osoittaa, että varmistustesti oli luotettava kalankasvatusvesissä.

Koliformisten bakteerien kokonaismäärä kasvoi kalankasvatuslaitoksilla samaan aikaan kuin varmistamattomien fekaalisten streptokokkien määrä, mutta huiput eivät ajoittuneet täsmälleen samanai-

kaisiksi. Koliformisten bakteerien lajiston tunnistaminen API 20 E tunnistusmenetelmällä osoittautui vaikeaksi, koska osa kannoista jäi tunnistamatta ja huomattava osa tunnistetuistakin lajeista poikkesi tyyppikannoista. Ympäristönäytteistä peräisin olevien koliformisten bakteerien on yleisestikin todettu sopivan huonosti lähinnä lääketieteellisten laboratorioiden luomiin tunnistusmenetelmiin ja bakteerisystematiikkaan. Ympäristöstä peräisin olevat kannat ovat muuttamassa bakteeritaksonomiaa. Numeerisen taksonomian menetelmien sekä kehitteillä olevien ympäristönäytteille tarkoitettujen tunnistusmenetelmien avulla näiden bakteereiden ekologiaa pystytään paremmin selvittämään.

Koliformisten bakteerien kokonaismäärästä kalankasvatuslaitoksilla vain pieni osa oli *E. coli*-lajia, mutta lämpökestoisista koliformisista bakteereista valtaosa oli tätä luotettavimpana ulosteiden aiheuttaman saastutuksen osoittajana pidettyä lajia. Yleensä lämpökestoisten koliformisten bakteerien määrät olivat tulevassa ja lähtevässä vedessä samanaikaisesti kohonneita.

Kuitenkaan niiden lisääntymistä satunnaisesti kalankasvatuslaitoksilla ei voitu sulkea pois. Satunnainen lisääntyminen voitiin selittää lintujen ulosteiden huuhtoutumilla altaiden penkereiltä sateella ja mahdollisesti likaantuneen tuorerehun käytöllä.

Molempien tutkittujen Rautalammin reitin varrella olevien kalankasvatuslaitosten ottaman veden todettiin sisältävän vähän *E. coli* -lajia. Saastutuksen määrä nousi sateisina aikoina ja keskellä kesää. Saastutuksen mahdollisia lähteitä ovat karjanlaitumilta tulevat valumavedet ja mahdollisesti kesämökkien aiheuttama kuormitus loma-aikoina.

Lämpökestoiset koliformiset bakteerit osoittautuivat tutkituissa olosuhteissa luotettaviksi ihmisen ja tasalämpöisten eläinten ulosteista mahdollisesti aiheutuvaa vaaraa osoittavaksi bakteeriryhmäksi kalankasvatuksen kuormittamilla vesialueilla.

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Appendix 1. Chemical and physical water characteristics at the Tyyrinvirta farm in 1981. The influent values are from one sample and the effluent values are flow weighted averages of two effluent fractions.

Variable	Sample	June		July		August		September	
		15th	29th	14th	28th	10th	25th	9th	22nd
Oxygen saturation %	I[1]	90	95	92	91	(115)	89	90	87
	E[2]	74	74	52	49	77	55	59	61
Total phosphorus $\mu g\ l^{-1}$ P	I	13	9	9	12	9	11	11	12
	E	51	71	100	109	75	96	96	83
Phosphate phosphorus $\mu g\ l^{-1}$ P	I	2		1	1	0	2	1	1
	E	19		48	56	30	59	53	45
Total nitrogen $\mu g\ l^{-1}$ N	I	505	431	392	378	327	425	372	294
	E	745	515	1 075	870	749	928	903	826
Chemical oxygen demand $mg\ l^{-1}\ O_2$	I	8.2	8.6	8.3	8.4	7.5	8.2	8.7	8.0
	E	8.8	8.8	9.0	9.6	8.0	9.2	9.9	8.8
pH	I	7.1	7.1	7.0	7.1	7.3	7.3	7.4	7.3
	E	6.7	6.6	6.6	6.6	6.7	6.7	6.7	6.6
Conductivity mS m^{-1}	I	4.5	4.4	4.5	4.4	4.3	4.4	4.4	4.4
	E	4.6	4.6	4.9	4.8	4.6	4.8	4.8	4.8

[1] Influent
[2] Effluent

Appendix 2. Chemical and physical water characteristics at the Tyyrinvirta farm in 1982. The influent values are from one sample and the effluent values are flow weighted averages of two effluent fractions.

Variable	Sample	June		July		August			September	October
		8th	21st	5th	19th	2nd	16th	30th	27nd	25th
Oxygen saturation %	I[1]	95	95	95	93	95	86	89	88	90
	E[2]	69	76	66	55	56	62	62	56	78
Total phosphorus $\mu g\ l^{-1}$ P	I	13	8	10	10	11	11	10	11	8
	E	55	51	96	48	54	47	56	67	36
Total nitrogen $\mu g\ l^{-1}$ N	I	490		360	370	420	310	380	390	380
	E	663		535	523	630	530	567	579	556
Ammonium nitrogen $\mu g\ l^{-1}$ N	I	5	6	6	10	15	9	53	9	15
	E	187	86	66	70	71	47	42	47	69
Chemical oxygen demand $mg\ l^{-1}\ O_2$	I	9.5	9.5	9.2	8.9	8.7	8.4	8.1	8.5	7.4
	E	9.8	10.4	9.7	9.1	9.4	8.8	9.0	8.7	8.0
pH	I	7.1	7.3	7.3	7.2	7.4	7.2	7.1	7.2	6.9
	E	6.7	6.8	6.6	6.6	6.7	6.6	6.5	6.5	6.6
Conductivity mS m^{-1}	I	4.4	4.2	4.1	4.4	4.4	4.4	4.4	4.4	4.4
	E	4.5	4.4	4.4	4.5	4.6	4.5	4.5	4.4	4.4

[1] Influent
[2] Effluent

Appendix 3. Chemical and physical water characteristics at the Äyskoski farm in 1981. The influent values are from one sample and the effluent values are flow weighted averages of two effluent fractions.

Variable	Sample	June		July		August		September	
		9th	22nd	7th	20th	4th	18th	1st	14th
Oxygen saturation %	I[1)]	96	93	91	86	89	85	87	89
	E[2)]	72	70	57	56	62	53	60	62
Total phosphorus $\mu g\ l^{-1}$ P	I	9	10	10	13	14	12	16	8
	E	72	69	88	101	82	89	98	83
Total nitrogen $\mu g\ l^{-1}$ N	I	434	443	462	434	397	551	584	420
	E	832	694	860	869	710	908	848	640
Chemical oxygen demand $mg\ l^{-1}\ O_2$	I	9.5	9.5	9.7	11.2	12.1	10.0	10.5	10.8
	E	10.9	10.5	10.8	12.0	11.8	11.5	11.5	11.5
pH	I	7.0	7.0	7.0	7.0	7.2	7.2	7.0	7.2
	E	6.5	6.5	6.4	6.5	6.5	6.6	6.4	6.5
Conductivity mS m^{-1}	I	4.2	4.1	4.0	4.1	4.0	4.1	4.2	4.0
	E	4.5	4.4	4.5	4.4	4.3	4.5	4.5	4.3

1) Influent
2) Effluent